

Appl. No. 09/775,046
Amdt. Dated August 11, 2003
Reply to Office Action of March 11, 2003

REMARKS

Claims 1-20 are pending. Claims 4-11 are rejected. Claims 1-3 and 11-20 are cancelled, without prejudice. The specification is amended to provide sequence identifiers. Support for this amendment can be found, e.g., on page 106, lines 1-13, of the specification. Claims 4-10 are amended. Support for this amendment can be found, e.g., on page 7, lines 4-6; page 70, lines 9-11; page 74, lines 4-26; and page 106, lines 3-17, of the specification.

Applicants thank the Examiner for rejoining the species: inflammation, proliferation, and tissue remodeling; and for rejoining the species: IL-1 ϵ antagonist alone and IL-1 ϵ antagonist with chemokine receptor antagonist.

Applicants believe that no new matter is added by way of amendment.

I. Objections to the Specification.

The Examiner objected to the lack of sequence identifiers in the brief description of the drawings (page 4, lines 9-14, specification), and to the partial receptor designation (page 77, line 34, specification). Applicants submit that the appropriate sequence identifiers are provided. Also, the partial receptor designation is cancelled. Withdrawal of the objections is respectfully requested.

II. Rejections of Claims 4-11 under 35 U.S.C. §112, First Paragraph.

The Examiner rejected Claims 4-11 under 35 U.S.C. §112, first paragraph on the basis that these claims are drawn to methods of inhibiting inflammation, but that one of skill would not "be able to predict that such a nexus [between IL-1R6 function and inflammation] exists." (page 4, lines 1-8, of Office action). Claim 11 is cancelled and the rejection is therefore moot as to this claim.

Applicants respectfully disagree that one of skill would not be able to predict a nexus between the present invention and inflammation. According to the MPEP, the test of enablement is whether "any person skilled in the art can make and use the invention without undue experimentation," in view of, e.g., the

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level of predictability in the art, or the amount of direction provided by the inventor (§2164.01-2164.01(a) MPEP, August 2001). Methods of modulating inflammation are fully enabled by the specification which discloses that, "[i]n lesional psoriasis skin, characterized by chronic inflammation, the expression of . . . IL-1 ϵ and their corresponding receptor IL-1R6 are all significantly increased relative to skin from a healthy individual. The increase is most prominent for IL-1 ϵ . . ." (See, e.g., page 70, lines 18-19; page 95, lines 13-17, of specification).

The level of predictability in the art at the time of filing is evidenced by Zweiger (1999) *Trends Biotechnol.* 17:429-436, who states that expression data is a likely or expected indicator of disease: ". . . gene transcripts that fall outside their normal range are likely to be indicative of particular disease states, diseases propensities or disease outcomes." (emphasis added)

In view of the increase of IL-1 ϵ in psoriasis, where this increase is more prominent than of the other molecules tested, and Zweiger's assertions that gene expression levels are a "likely" indicator of disease, Applicants conclude that one skilled in the art would reasonably believe that a nexus between the present invention and inflammation is established without undue experimentation.

Furthermore, the present invention modulates inflammation because the IL-1 ϵ is disclosed as relaying signals via NF κ B (page 4, lines 1-2, Office action; page 73, lines 27-29, specification). At the time of filing, it was established in the art that inflammation is mediated by NF κ B (see, e.g., page 70, lines 15-17, of specification; Lentsch, et al. (1999) *Inst. For Lab. Animal Res.* 40:151-156 (enclosed); Ward, et al. (1999) *Arch. Surg.* 134:666-669 (enclosed)). At the time of filing, it was also well established that psoriatic inflammation is mediated by NF κ B (see, e.g., page 1819 of Robert and Kupper (1999) *New Engl. J. Med.* 341:1817-1828; Komine, et al. (1999) *Arch. Dermatol. Res.* 291:500-506 (enclosed)). In view of the demonstration that the IL-1 ϵ /IL-1R6 signaling pathway transmits an activating signal to NF κ B, the established central role of NF κ B in inflammation, and the established role of NF κ B in psoriatic inflammation,

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Applicants conclude that one of ordinary skill would reasonably predict that a nexus exists between the present invention and inflammation without undue experimentation.

Further evidence that the present invention modulates inflammation is the fact that expression of IL-1R6 occurs on resident epithelial cells, constituting the ports of entry to the body's internal milieu (see, e.g., p. 70, lines 10-11, of specification), as well as on lung, an organ closely associated with inflammatory processes. Note that IL-1R6 is also known as IL-1 ϵ receptor and IL-1Rrp2. Expression of IL-1R6 is higher in lung than that in any other tissue tested (page 4, lines 9-10, of Office action; and Fig. 3A of Lovenberg, et al. (1996) *J. Neuroimmunol.* 70:113-122). Epithelial cells of the lung have been shown to be inflammatory because: "[a]irway epithelial cells . . . interact directly with T lymphocytes" and "epithelial cell[s] release . . . [G]-CSF . . . demonstrating that the epithellum has the potential . . . for attracting cells" (p. 451, column 1, of Crystal, et al. (eds) (1997) *The Lung*, Lippincott-Raven, NY, NY).

Moreover, one skilled in the art would believe that the present invention modulates inflammation because of the homology of the IL-1 ϵ (agonist) and IL-1R6 (receptor) signaling scheme to a previously described signaling scheme comprising IL-1 α (inflammatory agonist), IL-1R1 (receptor), and IL-1RA (antagonist) (see, e.g., page 10, lines 13-14; page 71, line 33, page 72, lines 1-3; page 77, lines 21-27; and page 78, lines 16-18, of the specification). Applicants conclude that the homologous actions of these two sets of highly similar signaling patterns establishes IL-1 ϵ as an inflammatory cytokine.

The Examiner further finds that IL-1R6 is not expressed in "cells associated with the immune system." (page 4, lines 10-11, Office action). Applicants respectfully disagree. Applicants submit that IL-1R6 is expressed in monocytes, a cell of the immune system (page 6, lines 1-2, of Office action; and page 79, lines 1-3, specification).

The Examiner cites WO 99/36541 of Marshall and Young, and three documents of Young (sole), as teaching that one of skill would expect that human

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IL-1 ϵ (a.k.a. IL-1 β) could treat inflammation, in contrast to the present claims, which encompass use of an IL-1 ϵ antagonist to treat inflammation (page 5, lines 8-9, of Office action). Applicants respectfully disagree. According to the MPEP, "the analysis and conclusion of lack of enablement are based on . . . findings of fact, supported by the evidence, and then drawing conclusions based on these findings of fact." (§2164.04 MPEP, August 2001). The Examiner states that "none of these documents provide working examples or other objective evidence of this molecule's [IL-1 ϵ] function . . ." (page 5, lines 3-4, of Office action). In view of this lack of "objective evidence" in the cited patent documents, Applicants conclude that the Examiner has not satisfied the requirement to base a conclusion of lack of enablement on "findings of fact, supported by the evidence." (§2164.04 MPEP, August 2001).

The Examiner further finds that the specification does not enable "antagonists other than antibodies or muteins" (page 5, lines 15-19, Office action). Applicants respectfully disagree. IL-1 δ is disclosed to antagonize IL-1 ϵ -mediated stimulation of IL-1R6 (page 74, lines 4-6 and 23-26, of specification). Amended Claims 4-5, 8, and 10, relate to compositions derived from antibodies and to IL-1 δ .

The Examiner alleges that the combination of a chemokine receptor antagonist and an IL-1R6 inhibitor would not have an inhibitory effect, in view of the alleged unpredictable effects of an IL-1R6 (page 6, lines 6-12, of Office action). Amended Claim 10 no longer recites "combination."

To summarize, Applicants submit that the skilled artisan would reasonably believe that a nexus between the invention and inflammation exists because of prominent expression of IL-1 ϵ in psoriasis, the demonstrated ability of the invention to relay a signal to NF κ B, and the established central role of NF κ B in psoriasis and other forms of inflammation. This is further supported by the known inflammatory activity of lung epithelial cells (Crystal, et al. (eds.), supra), and the elevated expression of IL-1R6 in epithelial cells, monocytes, and lung (p. 70, lines 10-11, and p. 79, lines 1-3, of specification; Lovenberg, et al., supra).

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and homology of the invention with an inflammatory signaling scheme comprising IL-1 α , IL-1R1, and IL-1RA. Moreover, the cited Marshall and Young and Young (sole) documents are believed not to detract from enablement of the present invention because of the lack of evidence in these documents (§2164.04 MPEP, August 2001).

Applicants believe the rejection of Claims 4-11 under 35 U.S.C. §112, first paragraph, is overcome. Withdrawal is respectfully requested.

III. Rejections of Claims 5-6 under 35 U.S.C. §112, Second Paragraph.

The Examiner rejected Claims 5-6 under 35 U.S.C. §112, second paragraph, alleging that these claims omit essential steps relating to "negative limitations."

Applicants submit that amended Claims 5 and 6 no longer recite the "negative limitations," thus rendering moot this basis for rejection.

Applicants believe the rejection of Claims 5-6 under 35 U.S.C. §112, second paragraph, is overcome. Withdrawal is respectfully requested.

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Conclusion


Applicants' current response is believed to be a complete reply to all the outstanding issues of the latest Office action. Further, the present response is a bona fide effort to place the application in condition for allowance or in better form for appeal. Accordingly, Applicants respectfully request reconsideration and passage of the amended claims to allowance at the earliest possible convenience.

Applicants believe that no additional fees are due with this communication. Should this not be the case, the Commissioner is hereby authorized to debit any charges or refund any overpayments to DNAX Deposit Account No. 04-1239.

If the Examiner believes that a telephonic conference would aid the prosecution of this case in any way, please call the undersigned.

Respectfully submitted,

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Enclosed:

1. Lentsch, et al. (1999) *Inst. for Lab. Animal Res.* 40:151-156 (8 pages, online version).
2. Ward, et al. (1999) *Arch. Surg.* 134:666-669 (4 pages).
3. Robert and Kupper (1999) *New Engl. J. Med.* 341:1817-1828 (12 pages).
4. Komine, et al. (1999) *Arch. Dermatol. Res.* 291:500-506 (7 pages).
5. Crystal, et al. (eds) (1997) *The Lung*, Lippincott-Raven, NY, NY, p. 451 (3 pages).



ILAR Journal Home

**ILAR Journal V40(4) 1999
Animal Models of Inflammation****Understanding the Pathogenesis of Inflammation Using Rodent Models****Identification of a Transcription Factor (NF κ B) Necessary for Development of Inflammatory Injury****Alex B. Lentsch and Peter A. Ward**

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Introduction

Acute inflammation is a necessary response to tissue injury, designed to maintain homeostasis by returning the tissue to its preinjury state. In general, this response can be characterized as a cascade of events that result in complex, yet coordinated, interactions between blood leukocytes, blood vessels, and cells of the tissue(s) involved. These events are directed toward removal of injurious agents and restoration of normal tissue structure and/or function. However, dysregulated inflammatory processes cause many human diseases. Thus, successful development of therapeutic strategies to suppress undesirable inflammatory responses depends on two factors: (1) knowledge of the steps leading to activation of the inflammatory response and (2) an understanding of the regulatory mechanisms that serve to control the progression and/or resolution of the inflammatory response. Much has been learned about these two factors using rodent models of inflammation.

Lung inflammatory injury induced in rats by distal airway deposition of immunoglobulin G (IgG¹) immune complexes has been used for many years for the study of lung inflammation (Johnson and Ward 1974). The inflammatory pathways in this particular model are rather similar to events related to lung injury caused by ischemia (Coty and others 1990), by the presence of bacteria (Lechner and others 1993), or by bacterial lipopolysaccharide (Simons and others 1996) and therefore represent a model relevant to human disease. In rats and mice, hepatic ischemia and reoperation results in both local and remote organ inflammation (Colletti and others 1990a; Jaeschke and others 1990; Lentsch and others 1998a). These models are clinically relevant because liver resectional surgery (Huguet and others 1994), liver transplantation (Lemasters and Thurman 1997), and hemorrhagic shock with fluid resuscitation (Vedder and others 1989) are all complicated by inflammatory organ injury stemming from ischemic insult to the liver.

The inflammatory responses in lung and liver models share many similarities (Figure 1). The inflammatory insult causes complement activation as well as activation of tissue macrophages (Jaeschke and others 1993; Ward 1996). Activated macrophages generate the "early response" cytokines, tumor necrosis factor (TNF¹)- α and interleukin (IL¹)-1 (Colletti and others 1990b; Mulligan and Ward 1992). These cytokines stimulate vascular endothelial cells to express adhesion molecules (ICAM-1, E-selectin), which facilitate adhesion of blood neutrophils to the endothelium (Springer 1990). TNF α and IL-1 also stimulate the production of neutrophil chemoattractants that are the chemokines of the IL-8 family of cytokines, from vascular endothelial cells and other tissue parenchymal cells (Schall and Bacon 1994). Vascular adhesion molecules and chemokines work in concert to bring about neutrophil transmigration from blood vessels to the tissue interstitium (Springer 1994). In organs such as lung and liver, the accumulation of neutrophils together with activated macrophages results in tissue injury mediated by the generation and release of oxidants and proteases (Jaeschke and others 1996; Varani and others 1989).

Regulation of Inflammatory Mediator Production by NF κ B

Recently, a considerable amount of research has been aimed at the upstream, molecular mechanisms that regulate gene expression of proinflammatory mediators. Using monocyte and macrophage cell lines in vitro, the transcription factor nuclear factor kappa B (NF κ B¹) has been shown to be a major regulator of many functionally diverse proinflammatory mediators. NF κ B is a general term used to describe a number of dimeric

combinations of members of the Rel family of gene regulatory proteins that possess transcriptional activating properties (Ghosh and others 1998). The most common form of NF κ B consists of a heterodimer of p50 (NF κ B1) and p65 (RelA) proteins. This complex has the ability to bind with promoter sequences in DNA and to inaugurate transcription (generation of mRNA) for many proinflammatory mediators. However, other combinations of Rel family members have been identified, and different configurations of Rel proteins (such as p65/p50 and p65/p52) may have preferential sensitivities to different target promoter sequences (Perkins and others 1992). In unstimulated cells, NF κ B is retained in the cytoplasm through interactions with inhibitory proteins of the inhibitory factor kappa B (I κ B¹) family. To date, at least seven I κ B proteins have been identified in vertebrates (Ghosh and others 1998). All I κ B proteins contain ankyrin repeat domains, which function to facilitate protein-protein interactions. In the case of I κ B/ NF κ B interactions, ankyrin repeat domains of I κ B proteins prevent nuclear translocation of NF κ B by masking nuclear localization sequences of the NF κ B (hetero)dimers. In response to a wide variety of cellular stimuli, I κ B proteins are dissociated from the NF κ B complex and then proteolytically degraded (Figure 2). This process varies for different I κ B proteins but involves phosphorylation of I κ B by members of the I κ B-kinase (IKK) family (DiDonato and others 1997). Phosphorylation targets I κ B for ubiquitination and degradation by the 26S proteasome. Degradation of I κ B leads to "activation" of NF κ B, which is defined as translocation of the NF κ B complex from the cytoplasm to the nucleus. Once in the nucleus, NF κ B binds specific promoter elements of DNA and induces transcription of relevant genes. The specificity of NF κ B for DNA promoter segments is dependent on nucleotide base sequences recognized by NF κ B. The mediators under the control of NF κ B include the proinflammatory cytokines TNF α and IL-1 (Collart and others 1990; Hiscott and others 1993), numerous chemokines (Widmer and others 1993), and many vascular endothelial cell adhesion molecules (Collins and others 1995). Obviously, the next logical step pursuant to the findings described above was to determine whether NF κ B was involved in inflammatory responses in vivo.

Use of Rodent Models to Delineate the Role of NF κ B during Inflammatory Reactions In Vivo

As outlined in Figure 1, the use of rodent models has allowed identification of many of the mediators involved in the development of acute inflammatory injury. The next objective was to apply the knowledge gained from in vitro studies of NF κ B to determine whether this transcription factor was involved in the inflammatory response occurring in complex organ systems. Initial studies in a rat model of systemic inflammation induced by intraperitoneal injection of bacterial lipopolysaccharide demonstrated that activation of NF κ B occurred in numerous tissues (Blackwell and others 1994; Essani and others 1996; Manning and others 1995). In addition, these studies showed that expression of chemokines and adhesion molecule mRNA was associated with NF κ B activation. Subsequent studies of more carefully controlled models of inflammation helped characterize the precise role of NF κ B during inflammation of different organs.

NF κ B Activation during Acute Lung Inflammation

Using a rat model of lung inflammation induced by intra-pulmonary deposition of IgG immune complexes, the precise time course of NF κ B activation during lung injury has been documented (Lentsch and others 1997, 1998b). In this model, alveolar macrophages (obtained by bronchoalveolar lavage) are activated by the inflammatory insult (that is, IgG immune complexes) and rapidly demonstrate increased nuclear translocation of NF κ B. The activation of NF κ B in alveolar macrophages is associated with enhanced production of the proinflammatory cytokines TNF α and IL-1 (Mulligan and Ward 1992). These cytokines are known to cause upregulation of chemokines and vascular adhesion molecules within the lung (Mulligan and others 1993; Shanley and others 1997). When either TNF α or IL-1 was neutralized using blocking antibodies, lung NF κ B activation was greatly attenuated (Lentsch and others 1998b). Furthermore, when alveolar macrophages were depleted using liposome-encapsulated dichloromethylene diphosphonate, lung NF κ B activation was virtually abolished (Lentsch and others 1998a). In rats depleted of alveolar macrophages, lung instillation of TNF α caused activation of NF κ B in whole lung tissues. These studies suggest that during acute inflammatory lung injury, activation of NF κ B in alveolar macrophages may be responsible for proinflammatory cytokine production. These proinflammatory cytokines appear to propagate the inflammatory response in lung by activating NF κ B in other lung cell types, possibly resulting in the expression of chemokines and vascular adhesion molecules in a variety of cell types.

The importance of NF κ B activation during lung inflammation has also been demonstrated in studies employing agents that specifically inhibit the nuclear translocation of NF κ B. In vitro, antioxidants prevent the phosphorylation and degradation of I κ B and limit the extent of nuclear translocation of NF κ B (Ghosh and

others 1998). In vivo administration of the antioxidant N-acetylcysteine suppressed lung NF κ B activation induced either by intraperitoneal injection of lipopolysaccharide or by intrapulmonary deposition of IgG immune complexes (Blackwell and others 1996; Lentsch and others 1998b). Interestingly, it was found that another antioxidant, catalase, was incapable of inhibiting lung NF κ B (Lentsch and others 1998b). Because N-acetylcysteine is a very small molecule (163 d) that easily diffuses across cell membranes and catalase is very large (~240 kDa) and probably does not gain cellular entry, it appears that only oxidants generated in the cytoplasm of lung cells are involved in the activation of NF κ B.

The use of antioxidants in the study of NF κ B is limited due to the relatively nonspecific nature of these agents. What is unclear in these studies is how the antioxidants affect NF κ B activation. Furthermore, these agents also reduce tissue injury by scavenging the oxidants released from activated phagocytic cells. Thus, information from these studies regarding the role of NF κ B in inflammatory injury is somewhat speculative. In contrast, other studies employing anti-inflammatory cytokines known to regulate the production of TNF α and IL-1 have suggested that NF κ B may be central to the acute inflammatory response. Two of the most potent anti-inflammatory cytokines, IL-10 and IL-13, greatly reduce lung inflammatory injury while almost completely suppressing activation of NF κ B (Lentsch and others 1997). Inhibition of NF κ B activation by both IL-10 and IL-13 was accomplished by preserving the cytoplasmic expression of the NF κ B-inhibiting protein I κ B β . These studies not only helped identify the in vivo anti-inflammatory mechanisms of IL-10 and IL-13, but they also increased our understanding of the regulation of lung inflammatory injury. Both IL-10 and IL-13 are constitutively expressed in lung, and endogenous production of these cytokines serves as a negative feedback loop of the inflammatory response, potentially limiting the progression of inflammation by inhibiting NF κ B activation (Lentsch and others 1999a). In other studies, a serine protease inhibitor, secretory leukocyte protease inhibitor (SLPI), was shown to suppress lung inflammatory injury as well as inhibit the activation of NF κ B (Lentsch and others 1999b). These inhibitory effects of SLPI were associated with upregulation of the NF κ B-inhibiting protein I κ B β . Blockade of endogenous SLPI with antibody augmented the lung inflammatory response and enhanced activation of NF κ B (Lentsch and others 1999b). These studies strongly suggest that endogenous IL-10, IL-13, and SLPI regulate the inflammatory response in vivo by their effects on NF κ B activation.

NF κ B Activation during Acute Liver Inflammation

The involvement of NF κ B in acute liver inflammation induced by hepatic ischemia and reperfusion in rats and mice has also been evaluated. In these models, hepatic ischemia causes activation of liver macrophages (Kupffer cells). These cells release reactive oxygen species and proinflammatory cytokines, including TNF α , which may directly injure liver parenchymal (hepatic) cells. However, enhanced production of TNF α plays a more important role in the initiation of a cascade of events leading to the later phase of liver injury, which is mediated by neutrophils (Figure 1). One of the main functions of TNF α is the hepatic upregulation of adhesion molecules and neutrophil-attracting chemokines (Colletti and others 1995, 1998; Lentsch and others 1998c). The coordinated actions of adhesion molecules and chemokines mediate the recruitment of neutrophils into the liver. Sequestered neutrophils release proteases and reactive oxygen intermediates, which directly damage hepatocytes and endothelial cells and also contribute to capillary plugging, causing hepatic hypoperfusion (Jaeschke and others 1996; Vollmar and others 1996).

Using this model, activation of NF κ B in the liver was shown to occur shortly after reperfusion (Bradham and others 1997; Yoshidome and others 1999; Zwacka and others 1998a). Although the details of cell-specific NF κ B activation have not yet been delineated in this model of inflammation, the time course of activation is consistent with upregulation of vascular cell adhesion molecules and chemokines within the liver (Colletti and others 1998; Lentsch and others 1998c). Similar to studies of lung inflammation, treatment with anti-oxidants reduced liver injury in association with suppressed activation of NF κ B (Zwacka and others 1998b). In addition, investigations of IL-10 and SLPI demonstrate that these anti-inflammatory mediators also reduce hepatic ischemia/reperfusion injury through effects on NF κ B (Lentsch and others 1999c; Yoshidome and others 1999). These effects are of interest because unlike the lung in which NF κ B activation is associated with degradation of I κ B α (Lentsch and others 1997, 1998b), activation of NF κ B during hepatic ischemia/reperfusion occurs without measurable degradation of either I κ B α or I κ B β (Zwacka and others 1998a). A possible explanation is that exogenously administered IL-10 or SLPI may augment production of I κ B proteins as a mechanism of their inhibitory effects on NF κ B. However, whether endogenous production of these mediators regulates NF κ B activation and liver inflammatory injury remains to be determined.

Conclusion

The use of rodent models of inflammation has allowed detailed investigation into some of the earliest events in the induction of the acute inflammatory response. Because transcription factors such as NF κ B control gene expression of mediators at every level of the inflammatory response (proinflammatory cytokines, chemokines, adhesion molecules), knowledge gained from work done in animal models offers valuable therapeutic potential. Furthermore, these models have provided information critical to a more complete understanding of the regulation of inflammatory processes. It is very interesting that antiinflammatory mediators as diverse in function as cytokines (IL-10 and IL-13) and a protease inhibitor (SLPI) may suppress inflammatory responses through effects on a single transcription factor (NF κ B). Investigations of these mediators have also been performed in vitro, and although IL-10 and IL-13 suppress NF κ B activation in monocytes and macrophages, SLPI does not (Lentsch and others 1999b). These types of findings illustrate the necessity for the use of rodents in inflammation research and emphasize the fact that in vitro studies are often inadequate for a reflection of the in vivo response.

¹ Abbreviations used in this article: IgG, immunoglobulin G; Ir, B, inhibitory factor kappa B; IL, interleukin; NF- κ B, nuclear factor kappa B; SLPI, secretory leukocyte protease inhibitor; TNF α , tumor necrosis factor- α .

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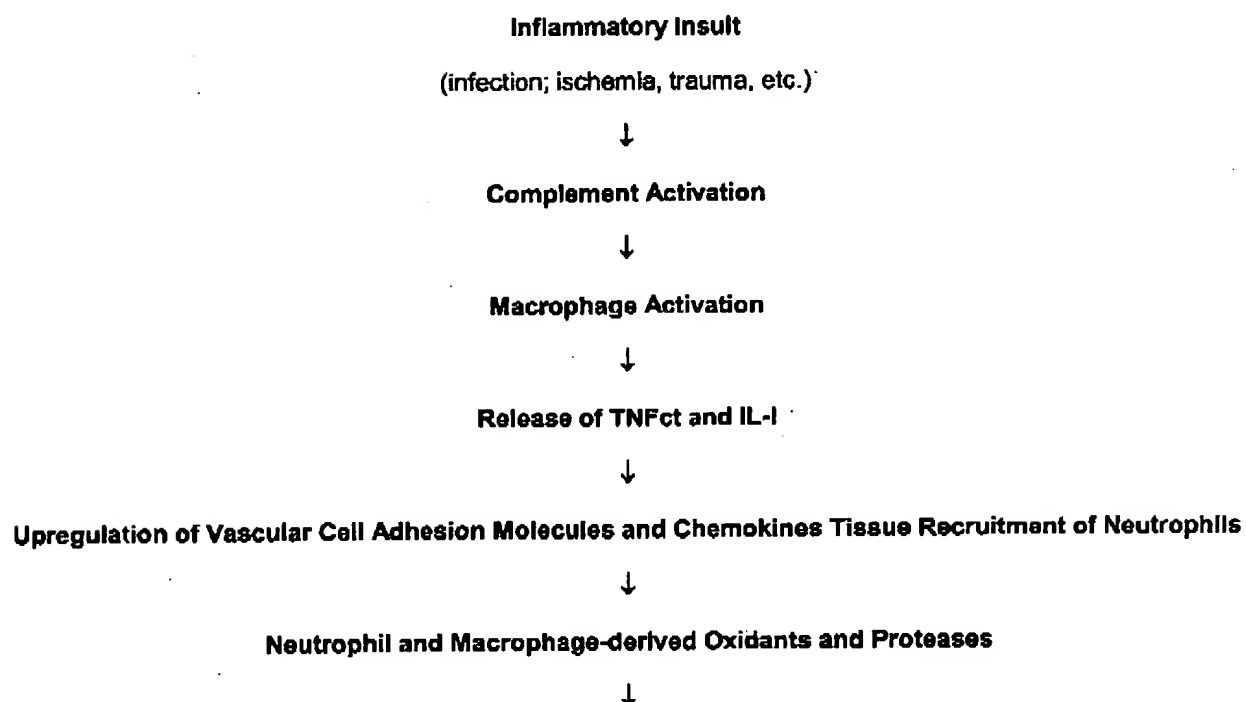
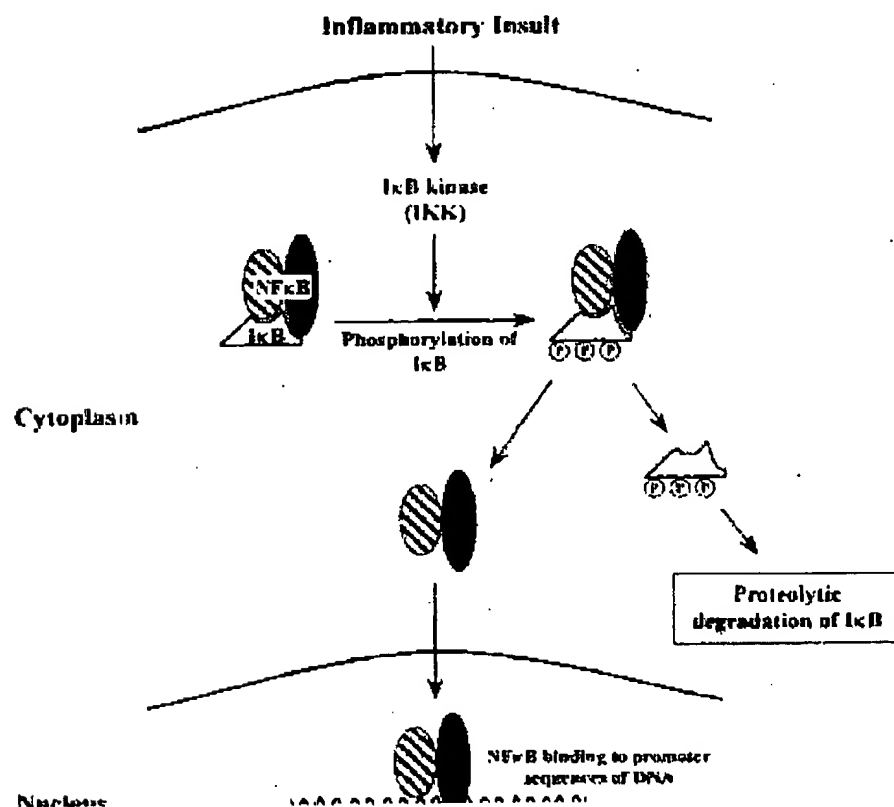


Figure 1 Common pathway of acute inflammatory tissue injury. TNF α , tumor necrosis factor- α ; IL, interleukin.



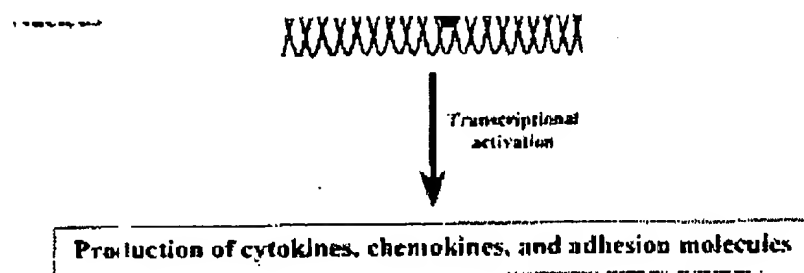


Figure 2 Mechanism of NFκB activation. IκB, inhibitory factor kappa B; NFκB, nuclear factor kappa B.

Close

BASIC SCIENCE FOR SURGEONS

The Acute Inflammatory Response and Its Regulation

Peter A. Ward, MD; Alex B. Lentsch, PhD

The acute inflammatory response is composed of an elaborate cascade of both proinflammatory and anti-inflammatory mediators. The balance between these mediators often determines the outcome after injury. In clinical scenarios, such as trauma or sepsis, there is often unregulated production of proinflammatory mediators that can cause multiple organ failure. Further understanding of the endogenous mechanisms that control the inflammatory response is needed to facilitate development of therapeutic options. In this review, we discuss the current knowledge of the mechanisms leading to development of acute inflammatory injury as well as the factors that regulate this response.

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The inflammatory process is a vital response to injury, infection, trauma, and many other insults. For a successful outcome after injury (including surgically induced trauma), the inflammatory response must be triggered to bring about recruitment of blood leukocytes, activation of tissue macrophages, and production of a series of mediators. The results of this may include ultimate resolution of the inflammatory process, triggering events that lead to cell regeneration and wound healing, or progression of the inflammatory response, which often leads to progressive organ dysfunction. Understanding how the inflammatory process is activated and how it is contained are key to developing strategies designed to block or reduce inflammatory responses, similar to immunosuppressive interventions when immune responses are unwanted (eg, allograft rejection) or exaggerated (eg, autoimmune responses). A good example of an undesirable inflammatory response occurs in the "systemic inflammatory response syndrome" during sepsis. In this situation, cytokines (eg, interleukin 1 [IL-1], IL-6, tumor necrosis factor α [TNF- α]) are detectable in the plasma, suggesting unregulated generation of these highly inflammatory peptides.¹ Under such conditions multiorgan failure often occurs. What remains to be determined is why, during sepsis, there is uncontrolled production of

cytokines and how these cytokines may be involved in multiorgan failure.

THE INFLAMMATORY CASCADE AND NEUTROPHIL RECRUITMENT

Much of our work, which has provided information about mediation of the acute inflammatory process, has occurred in the context of acute inflammation in lungs of rats. For convenience, these reactions are triggered by distal airway deposition of IgG immune complexes, which trigger complement activation and macrophage activation that ultimately result in large accumulations of neutrophils, interstitial and intra-alveolar edema, and intra-alveolar hemorrhage, each of which can be precisely quantitated.² The general scheme of the inflammatory response is outlined in **Figure 1**. The inflammatory pathways in this particular model are very similar to events related to lung injury caused by ischemia,³ by the presence of bacteria,⁴ or by bacterial lipopolysaccharide.⁵ These events trigger complement activation as well as activation of tissue macrophages. Activated macrophages generate the "early response cytokines," TNF- α and IL-1. A chief function of these cytokines is to stimulate vascular endothelial cells to express vascular adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and E-selectin.⁶ Through a series of adhesion-promoting events, blood neutrophils adhere intermittently to endothelial cells (the "rolling" phenomenon), followed by firm attachment and transmigration into the

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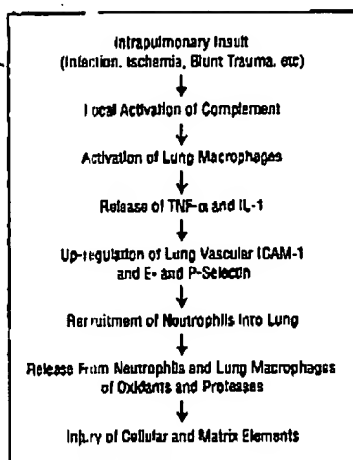


Figure 1. Model of acute inflammatory lung injury induced by intrapulmonary deposition of IgG immune complexes. TNF- α indicates tumor necrosis factor α ; IL-1, interleukin 1; and ICAM-1, intercellular adhesion molecule 1.

interstitial and alveolar compartments. The intermittent attachment phenomenon features endothelial selectin interactions with "counter-receptors" on neutrophils that contain oligosaccharides with the sialyl Lewis^x motif.⁷ Firm attachment of neutrophils to the endothelium involves endothelial ICAM-1 interactions with neutrophil $\beta 2$ integrins (CD11a/CD18 and CD11b/CD18).⁹ Activated endothelial cells also express platelet-activating factor and IL-8, powerful neutrophil-stimulating agonists.⁹ Accordingly, when neutrophils adhere to the activated endothelium, they also become activated or "primed," so that arrival at an extravascular site containing a neutrophil agonist such as TNF- α causes an exaggerated functional response in these neutrophils. The entry of neutrophils into the alveolar compartment together with tissue-activated macrophages sets the stage for injury of both lung cells and matrix glycoproteins (eg, collagens, elastin). Injury is related to generation of oxidants by phagocytic cells (described below) and the release of proteases (serine proteases and matrix metalloproteases).¹⁰

OXIDANT-GENERATING PATHWAYS

Most oxidants generated during the inflammatory response derive from phagocytic cells (neutrophils, mac-

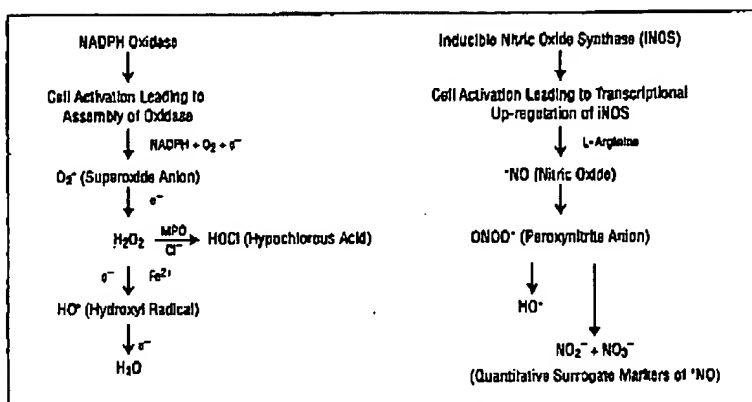


Figure 2. Mechanisms of oxidant production by activated phagocytic cells. NADPH indicates nicotinamide adenine dinucleotide phosphate (reduced form).

rophages, and monocytes) and are released into the extracellular environment, in part at least because one of the oxidants (nicotinamide adenine dinucleotide phosphate [NADPH] oxidase) is assembled in an enzymatically active form on surfaces of phagocytic cells. As shown in **Figure 2**, the 2 chief oxidant-generating pathways include NADPH oxidase and inducible nitric oxide synthase.^{11,12} The former enzyme exists as inactive subunits that are located both on the cell membrane and in the cytosol. Cell activation causes translocation of cytosolic subunits to the cell membrane, resulting in a multimeric complex that exhibits oxidase activity. The pathway of oxidant generation by NADPH oxidase is described in **Figure 2** and is characterized by an unusual series of single (rather than double) additions of electrons. In the presence of the oxidase, NADPH undergoes oxidation. The released electrons interact with molecular oxygen to cause its reduction, forming superoxide anion (O_2^-). One function of O_2^- is to reduce intracellular iron, converting Fe^{3+} to Fe^{2+} . A further electron addition to O_2^- converts it to hydrogen peroxide (H_2O_2), which can be further reduced to the most active of all oxygen-centered radicals, the hydroxyl radical (HO^\bullet). Generation of hydroxyl radical requires a heavy metal (such as iron) in its transition (unstable) state (Fe^{2+}). In giving up an electron to hydrogen peroxide, Fe^{2+} is reoxidized to Fe^{3+} . Hydroxyl radical is a highly reactive and damaging radical. If it is fur-

ther reduced, the product is water. In the context of phagocytic cells, such as neutrophils, release of myeloperoxidase in the presence of a halide, such as chloride (Cl^-), will enzymatically convert hydrogen peroxide to hypochlorous acid, another potent oxidant.

A second major oxidant-generating pathway in phagocytic cells involves inducible nitric oxide synthase, which is typically not expressed in resting (nonstimulated) cells, especially macrophages. On cell activation, however, inducible nitric oxide synthase is transcriptionally up-regulated, reacting with L-arginine to generate nitric oxide (NO), which relaxes smooth muscle cells and is mildly reactive with aromatic amino acids to form stable adducts, such as nitrotyrosine. These modifications of tyrosine-containing proteins have often been found at sites of inflammation. Depending on the protein and the position of the tyrosine residue, nitrosylation may impair protein function. Nitric oxide is converted to peroxynitrite anion ($ONOO^-$), which is highly reactive with thiol groups. Peroxynitrite anion can be further converted to hydroxyl radical in the absence of the requirement for a heavy metal cation. Finally, peroxynitrite anion is ultimately broken down into NO_2^- and NO_3^- , which serve as convenient quantitative surrogate markers of nitric oxide.

These 2 oxidant-generating pathways in phagocytic cells account for many tissue-damaging outcomes of inflammatory responses and may well impair physiological

responses to injury. Oxidants may perturb phagocytic cells to inappropriately generate mediators, such as cytokines and chemokines. While chemically derivatized versions of L-arginine effectively antagonize the ability of inducible nitric oxide synthase to react with its natural substrate (L-arginine), the *in vivo* use of such compounds leads to problems, because they also antagonize constitutive nitric oxide synthase of endothelial cells, leading to a loss in the regulation of vascular smooth muscle tone, resulting in systemic hypertension. There are, to our knowledge, no reliable, specific, and effective inhibitors *in vivo* for either NADPH oxidase or inducible nitric oxide synthase.

MECHANISMS OF PHAGOCYTIC CELL ACTIVATION

Since phagocytic cells, especially macrophages, are key sources of inflammatory mediators (such as cytokines and chemokines), understanding their activation process is important if inhibition of their mediator generation is to be successful. When a macrophage is activated (eg, after contact with bacteria, bacterial lipopolysaccharide, TNF- α , or many other agonists), a series of intracellular events leads to transcriptional activation of the cell (**Figure 3**). A heterodimeric complex termed nuclear factor κ B (NF- κ B) contains 2 subunits, most often p50 and p65.¹³ This complex has the ability to bind with promoter sequences in DNA and to inaugurate transcription (generation of messenger RNA) for many inflammatory peptides. However, the NF- κ B complex is held in check in the cytosol by inhibitors of the inhibitor κ B (I κ B) family, which bind to the NF- κ B complex and prevent its entry into the nucleus (translocation) and subsequent binding to DNA.¹⁴ Typically, when the macrophages are activated, I κ B undergoes phosphorylation and ubiquitination. Those changes set the stage for the 26S proteasome to enzymatically cleave I κ B. The liberated NF- κ B complex can then translocate to the nucleus, engage DNA-promoter sequences, and cause transcriptional up-regulation of mediators such as

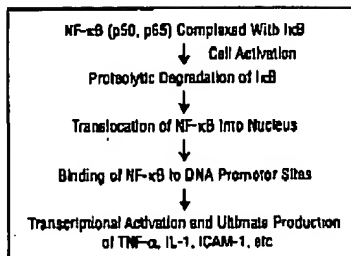


Figure 3. Pathways leading to activation of nuclear factor κ B (NF- κ B). I κ B indicates inhibitor κ B; TNF- α , tumor necrosis factor α ; IL-1, Interleukin 1; and ICAM-1, Interleukin adhesion molecule 1.

TNF- α , IL-1, and ICAM-1. Details of the NF- κ B pathway are especially relevant to understanding how the inflammatory system is regulated by certain interleukins.

REGULATORY INTERLEUKINS

As described above, the experimental model of IgG immune complex-induced acute alveolitis is known to be self-regulated. After 4 hours, there is no further progression in the albumin leak, cessation in further neutrophil recruitment, and rapid disappearance of mediators such as TNF- α . There is evidence that these inflammatory reactions also initiate the appearance of a series of regulatory cytokines that prevent continuation of the inflammatory response.

Regulatory ILs were originally discovered by their ability to inhibit cytokine (TNF- α) generation in macrophages stimulated *in vitro* with agonists such as lipopolysaccharide. Several of these ILs also inhibit *in vitro* T-cell responses. Animals that are unable to express IL-10 ("IL-10 knockout" mice) develop a progressive chronic inflammatory bowel disease similar to that in found in ulcerative colitis.¹⁵ Interleukin 10 knockout mice are also reported to be unable to contain a variety of other inflammatory responses (acute inflammation, delayed type hypersensitivity, etc). Using the model of immune complex-induced alveolitis in rats, several ILs were found to have a strong anti-inflammatory response, the rank (from most potent to least potent) being IL-10 = IL-13 > IL-4 > IL-6 > IL-12.¹⁶ The addition of exogenous ILs to rat lung caused reduced generation of TNF- α

in lung, which was associated with greatly reduced up-regulation of lung vascular ICAM-1, leading to reduced accumulations of neutrophils and diminished injury of the lung. The anti-inflammatory effects of some of these ILs extend to other inflammatory responses. For instance, virally induced *in vivo* expression of IL-10 or delivery of IL-10 by osmotic pumps greatly suppresses rejection of allografted hearts in mice and rats.^{17,18} The molecular mechanisms to explain these inhibitory outcomes are described below.

ROLES OF ENDOGENOUS REGULATORY ILs

The rat lung inflammatory model described above was used to assess the role of endogenous ILs. Complementary DNA for candidate ILs were cloned in the rat, the proteins were expressed, and blocking antibodies were developed. Assessing candidate endogenous regulatory ILs required the ability to demonstrate expression in lung of messenger RNA and protein for a given IL. In the most critical part of these studies, animals were treated with blocking antibody to the appropriate IL and the inflammatory response was quantitated. If a regulatory IL were blocked *in vivo*, then the expected outcome of the inflammatory response would be increased production in lung of TNF- α , increased expression of lung vascular ICAM-1, increased neutrophil accumulation in lung, and enhanced evidence of lung injury. Studies have identified the appearance of at least 3 regulatory ILs in the lung inflammatory response: IL-6, IL-10, and IL-13.¹⁹⁻²¹ Blockade of any 1 of these 3 ILs increased TNF- α levels in lung and caused an increase of at least 50% in the number of neutrophils recruited into lung.

Another regulatory factor identified in these studies was the IL-1 receptor antagonist (IL-1ra). IL-1ra is known to be a product of stimulated macrophages. It is often released *in vitro* following macrophage production of TNF- α and IL-1 and functions as a receptor "decoy," binding to the IL-1 receptor without triggering a cell response, thus competing with the ability of IL-1 to

bind to its natural receptor and to trigger signal transduction events. In the lung inflammatory model, both messenger RNA and protein for IL-1ra were found in lung tissues. Indeed, the protein could be detected both in macrophages and in neutrophils recruited into the lung. Antibody-induced blockade of endogenous IL-1ra, as with anti-IL-10, increased neutrophil accumulation by nearly 200%, notably increased the degree of extravascular albumin leak, and caused a nearly 2-fold increase in bronchoalveolar lavage levels of IL-1 β (but did not affect bronchoalveolar lavage levels of TNF- α).²² These data fairly convincingly indicate that endogenous IL-1ra is another regulator of the inflammatory response, at least in the lung.

MECHANISM OF REGULATION OF THE INFLAMMATORY RESPONSE

How regulatory ILs (eg, IL-10, IL-13) regulate the inflammatory response has been determined. As indicated in Figure 3, when the inflammatory response is triggered, there is in cytosolic extracts a rapid and profound loss of I κ B because of its hydrolysis by the 26S proteasome. This allows translocation of NF- κ B to the nucleus, where gene activation occurs. In the presence of IL-10 or IL-13, activation of NF- κ B fails to take place and the generation of inflammatory mediators is accordingly suppressed.²³ The striking finding was that IL-10 or IL-13 prevented the loss of I κ B in the inflamed tissue. In other words, breakdown of I κ B failed to occur, for reasons that are not known. There was no evidence that either IL-10 or IL-13 cause transcriptional up-regulation (appearance of messenger RNA) of I κ B. Retention of I κ B prevented the translocation of NF- κ B to the critical binding sites on DNA. The failure in breakdown of I κ B may be related to the ability of IL-10 or IL-13 to interfere with the phosphorylation and ubiquitination of I κ B, or interference in the enzymatic activity of the 26S proteasome. Whatever the explanation, IL-10 and IL-13 seem to

inhibit fundamental mechanisms leading to signal transduction and gene activation in pathways of the inflammatory response. It remains to be determined to what extent in vivo inhibition of NF- κ B activation will represent a new approach for anti-inflammatory therapy.

CONCLUSIONS

The acute inflammatory response is an essential and protective response in injured tissues; when successful, it restores the tissues to their preinjury state. On the other hand, there are many diseases and syndromes in which the inflammatory response produces adverse and sometimes life-threatening outcomes. In sepsis, it seems that the inflammatory response is no longer regulated, causing the appearance systemically of a variety of proinflammatory cytokines. These mediators cause expression of vascular adhesion molecules that facilitate the recruitment of blood leukocytes, especially neutrophils. Injury resulting from the inflammatory response is due to phagocytic cell production of oxidants and proteases. Central to generation of inflammatory mediators is activation in phagocytic cells of NF- κ B. The inflammatory response is naturally regulated by a variety of endogenous factors, including IL-10 and IL-13. These ILs suppress the inflammatory response by blocking activation of NF- κ B. The data suggest that a novel approach to inhibition of the inflammatory response would be to suppress the activation of NF- κ B in vivo.

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MECHANISMS OF DISEASE

*Mechanisms of Disease*FRANKLIN H. EPSTEIN, M.D., *Editor***INFLAMMATORY SKIN DISEASES,
T CELLS, AND IMMUNE
SURVEILLANCE**

CAROLINE ROBERT, M.D., AND THOMAS S. KLUPPER, M.D.

SKIN is the primary interface between the body and the environment. The spectrum of insults to which skin is susceptible includes disorders caused by chemical and microbial agents, thermal and electromagnetic radiation, and mechanical trauma. The most damaging consequence of the disruption of skin is invasion by pathogenic microorganisms, and the need for an effective means of protection against this challenge has been a fundamental force behind the evolution of the immune system. The translation of insults into cutaneous inflammation (innate immunity) and the recruitment of memory T lymphocytes that have clonally expanded in response to antigens encountered at the cutaneous interface with the environment (acquired immunity) are both required for successful cutaneous immune surveillance.

Certain memory T cells appear to remember the anatomical site where they first encountered antigen. Specifically, there is an identifiable subgroup of memory T cells with the ability to circulate preferentially to the skin. These memory T cells, identified by a marker known as cutaneous lymphocyte antigen (CLA),¹ are generated in lymph nodes draining skin and are recruited back to the skin during inflammation. Although their primary function is cutaneous immune surveillance, CLA-positive T cells have been implicated in the pathogenesis of relatively rare skin diseases, such as cutaneous T-cell lymphoma¹ and graft-versus-host disease after allogeneic bone marrow transplantation.² CLA-positive T cells also mediate many common skin diseases, including allergic contact dermatitis, psoriasis, atopic dermatitis, alopecia areata, vitiligo, drug-related eruptions, and lichen planus.³ The patterns of T-cell movement and migration that mediate cutaneous immune surveillance are central to an understanding of the clinical and pathological features of T-cell-mediated skin diseases.

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T CELLS AND IMMUNE SURVEILLANCE

Whereas antibodies recognize three-dimensional conformations of macromolecules, T-cell antigen receptors recognize antigens as fragments of macromolecules bound to antigen-presenting proteins on the surface of antigen-presenting cells. These cell-surface proteins include class I (HLA-A, B, and C) and class II (HLA-D) major histocompatibility complex molecules, which bind peptide antigens for presentation to CD8+ and CD4+ T cells, respectively,^{4,5} and CD1 molecules, which bind nonpeptide antigens for presentation to a different subgroup of T cells.^{4,5} T-cell antigen receptors are heterodimeric proteins composed of α/β or γ/δ chains. These receptors are encoded by four genes containing a large number of discrete genetic elements that recombine during intrathymic differentiation, generating an almost unlimited repertoire of T-cell receptors, each with a unique specificity.⁶ This great diversity is both a strength and a weakness. Although there may be a T-cell antigen receptor that is specific for every possible peptide antigen from a pathogen, establishing conditions in vivo under which a T cell will encounter the antigen for which its unique antigen receptor is specific represents a substantial logistic challenge.

The migratory behavior of T cells allows the immune system to overcome this logistic challenge. T cells that have never been activated by antigen (naïve T cells) efficiently migrate from blood into lymph nodes^{3,7} and return to blood through efferent lymphatics. The mechanisms by which these T cells enter lymph nodes from blood involves specific combinations of adhesion molecules and chemokines on specialized postcapillary venules in the endothelium (high endothelial venules), as well as L-selectin and other adhesion molecules and chemokine receptors on the T cells.^{8,9} Naïve T cells lack the specific combinations of adhesion molecules and chemokine receptors required to enter extranodal tissues from blood (Fig. 1).

The presentation of antigen to T cells, which is necessary for their activation, requires both the binding of the antigen-HLA or antigen-CD1 complex with the T-cell antigen receptor and additional costimulatory signals delivered by the antigen-presenting cells. Dendritic cells are specialized antigen-presenting cells that express high levels of costimulatory molecules and are uniquely capable of activating naïve T cells in lymph nodes. Skin contains large numbers of dendritic cells, in both the epidermis (Langerhans' cells) and the dermis.^{11,12} Macromolecules (including those derived from microorganisms) introduced after the skin has been disrupted are efficiently internalized by dendritic cells. After enzymatic processing in the endosomes of these cells, the antigens are bound to antigen-presenting molecules, and the resulting complex is expressed on the cell surface for presentation to T cells.^{5,11-13} These dendritic cells

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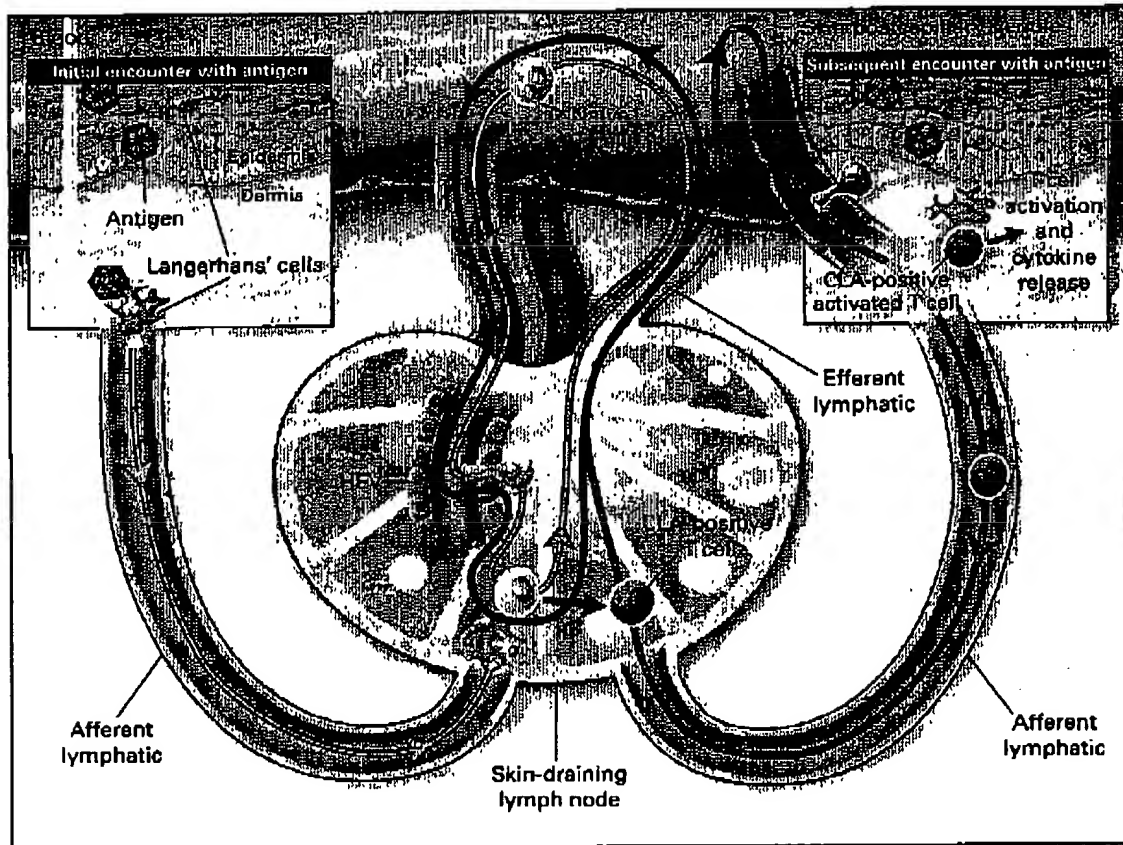


Figure 1. Movement of Previously Unactivated T Cells and CLA-Positive, Skin-Homing Memory T Cells.

T cells that have not previously been activated (naïve T cells) are continuously recirculating between blood and lymphoid organs. These cells express high levels of L-selectin, allowing them to attach to and roll on the surface of the high endothelial venules (HEV) in lymph nodes. The rolling T cells are activated by the secondary lymphoid-tissue chemokine,¹⁹ which is constitutively expressed on the luminal surface of the venules. The T cells are then activated by the chemokine receptor CCR7,¹⁹ which allows them to bind tightly to intercellular adhesion molecule 1 in the venules through lymphocyte functional antigen 1. The T cells can then extravasate through the HEV into the lymph node, where they accumulate in areas that are already rich in T cells. They then exit the node through efferent lymphatics and return to the blood. This pattern of movement is represented by the blue loop in the figure. Antigen-presenting cells that reside in the skin, such as Langerhans' cells and dermal dendritic cells, internalize foreign antigens in the skin and migrate to the lymph nodes through afferent lymphatics. When a naïve T cell encounters the antigen for which it is specific on an antigen-presenting cell in a skin-draining lymph node, it is activated and becomes a memory T cell (black arrow), with the expression of cutaneous lymphocyte antigen (CLA) and a distinct (although undefined) set of chemokine receptors. Endowed with these skin-homing molecules, CLA-positive T cells possess the molecular keys that allow them to migrate to skin, the site where the antigen was first encountered by the host. At least some of these CLA-positive memory T cells retain the capacity to exit HEV and enter the lymph node. The red loops in the figure represent the two possible patterns of movement of memory T cells.

migrate through afferent lymphatics and collect in lymph nodes replete with naïve T cells that have recently entered the lymph nodes from the blood. In this fashion, antigens derived from a large surface area of skin are concentrated at a single specialized site (the lymph node), where they come into contact with naïve T cells, making it likely that an interaction between antigen and T-cell antigen receptor will occur (Fig. 1).

Once these T cells have been activated by an antigen, they proliferate and express activation molecules, undergoing the transition to memory T cells.^{2,24} During this transition, the T cells acquire new molecular keys that allow them to exit the blood vessels in extranodal tissues.^{2,15} Lymph nodes that drain different epithelial interfaces with the environment (e.g., skin and the gastrointestinal tract) generate phenotypically distinct memory T cells that can exit the ves-

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sels at these specific extranodal sites.⁷ The best-studied T-cell component mediating this phenomenon is CLA, a glycoprotein molecule first expressed during the transition of T cells from previously unactivated cells to memory cells in lymph nodes that drain the skin.⁸ The expression of CLA by T cells involves the induction of glycosylation enzymes that modify a pre-existing protein (P-selectin glycoprotein ligand 1) in a highly specific fashion.¹⁶ Thus, memory T cells in inflammatory skin diseases express CLA on their surface; in contrast, T cells in inflammatory diseases involving tissues other than skin are predominantly CLA-negative.⁹

CLA is more than just a marker that identifies skin-specific T cells. It is an adhesion molecule that mediates the initial tethering of T cells to the endothelium in cutaneous postcapillary venules.^{15,16} This step is required for the subsequent slowing, arrest, and extravasation of the T cells, allowing them to overcome the substantial forces exerted by blood flow.^{15,16} E-selectin, the endothelial ligand for CLA, is expressed constitutively at low levels on cutaneous microvessels, but its expression is strongly up-regulated during cutaneous inflammation.¹⁷ The preferential expression of E-selectin in skin helps select for CLA-positive T cells under both normal and inflammatory conditions. Although interactions between CLA and E-selectin are required as the initial step in the extravasation of T cells from the blood into the skin, the activation of T cells through chemokines and the firm adhesion of T cells to the endothelium through interactions between integrin and cell adhesion molecules are also required¹⁸ (Fig. 2). The expression of unique chemokine receptors by CLA-positive T cells and the preferential expression of their respective chemokine ligands by skin cells increase the specificity of these T cells for skin.¹⁹

CUTANEOUS INFLAMMATION, CYTOKINES, AND NUCLEAR FACTOR- κ B-MEDIATED PATHWAYS

Interleukin-1 and tumor necrosis factor α , which have been called primary cytokines, have broad effects that are relevant to inflammation and immunity.²⁰ The epidermis is a storehouse of interleukin-1 α and can produce large amounts of interleukin-1 β and tumor necrosis factor α .^{20,21} After binding to their receptors, these cytokines activate several cellular signaling pathways, including the nuclear factor- κ B (NF- κ B) pathway.²² Among the many genes regulated by NF- κ B in skin cells, those that are central to the initiation of cutaneous inflammation include the genes for E-selectin, chemokines and cytokines, defensins (antibacterial peptides), intercellular adhesion molecule 1, and vascular-cell adhesion molecule 1.²²

Cytokines are not the only means of inducing NF- κ B responses in skin. Plants, insects, and mammals share a family of innate immune-cell surface re-

ceptors that signal through NF- κ B (or its plant and insect homologues).²³ In humans, these are known as Toll-like receptors (receptors that resemble the drosophila Toll protein).²⁴ Rather than binding cytokines, these receptors recognize conserved molecules derived from microbes; Toll-like receptor 2 was recently identified as a signal-transducing receptor for gram-negative bacterial lipopolysaccharide, as well as gram-positive bacterial lipoteichoic acid.^{25,26} Although their extracellular ligands are different, Toll-like receptors use multiple intracellular molecular elements in common with primary cytokine receptors, culminating in the translocation of NF- κ B to the nucleus²² and the transcription of genes that play an important part in cutaneous inflammation (Fig. 3). Many Toll-like receptors have been described, but the ligands for most of them are unknown.²³

NF- κ B-mediated inflammation in skin appears to be a final common pathway for the translation of environmental insults into inflammation and is a crucial element of innate immunity (Fig. 3). Even ultraviolet radiation from sunlight induces ligand-independent clustering and activation of interleukin-1 and tumor necrosis factor receptors,²⁷ leading to NF- κ B-mediated inflammation.

CLA-POSITIVE T CELLS AND CUTANEOUS INFLAMMATION

CLA-positive T cells represent 10 to 15 percent of all circulating T cells in peripheral blood, and although they have some features in common (e.g., their expression of CLA and certain chemokine receptors), their T-cell antigen-receptor specificities are quite heterogeneous. Furthermore, CLA-positive T cells may be positive for either CD4 or CD8, and once activated, they may be capable of producing either type 1 T-cell cytokines (interferon- γ , interleukin-2, and lymphotoxin) or type 2 T-cell cytokines (interleukin-4, 5, 10, and 13). This heterogeneity of phenotype and function is likely to be important for a successful and flexible host response to the plethora of distinct pathogens encountered in skin.

How do insults to the skin trigger immune surveillance and immunity mediated by CLA-positive T cells? NF- κ B transcriptional activation induces inflammation,²² favoring the recruitment of CLA-positive T cells to skin through E-selectin, chemokines, and cell adhesion molecules. Thus, cutaneous inflammation preferentially recruits memory T cells that have been activated by skin-related antigens. Because circulating CLA-positive T cells have previously encountered antigens in lymph nodes draining skin,³ this mechanism of immune surveillance mediated by memory T cells is based on the principle that common things occur commonly — in this case, that antigens encountered previously in skin may be responsible for (or at least associated with) the new insult.

Extravasation of CLA-positive T cells into skin does

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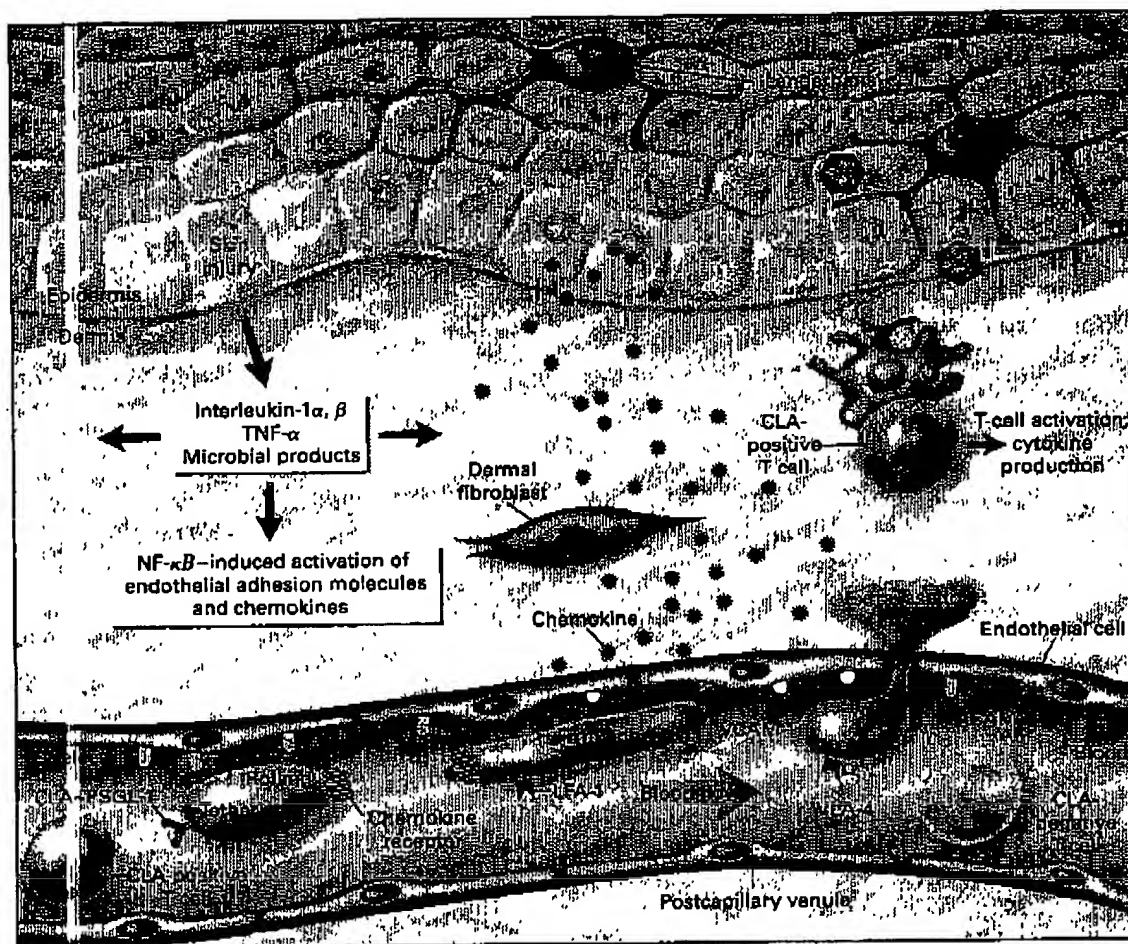


Figure 2. Extravasation of a CLA-Positive Memory T Cell into Inflamed Skin.

Skin injury or infection results in the activation of the nuclear factor- κ B (NF- κ B) pathway through cytokine receptors (Interleukin-1 or tumor necrosis factor α [TNF- α]) or Toll-like receptors. Microbial products may directly activate this pathway. The result is the transcription of many genes that contain κ B sites in their promoters in a variety of skin cells. In endothelial cells, these include the adhesion molecules E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular-cell adhesion molecule 1 (VCAM-1). To extravasate into skin, T cells must slow their velocity in the circulation. To do so, they use CLA-P-selectin glycoprotein ligand 1 (CLA-PSGL-1) cell-surface molecules, located on the tips of microvilli, to bind to E-selectin and P-selectin on the luminal surface of the cutaneous postcapillary venules, a process called "tethering." Once tethered, the T cells roll on the endothelial surface in the direction of the blood flow, but much more slowly. This exposes much of the surface of the T cells to the surface of the endothelium, where chemokines that have been produced on the abluminal side of the vessel by resident skin cells and transported to the luminal surface of the endothelial cells can be displayed. The binding of chemokines to specific receptors on T cells results in a modification of the structure of the $\alpha_L\beta_2$ integrin (lymphocyte-function-associated antigen 1 [LFA-1]) and the $\alpha_E\beta_7$ integrin (very late antigen 4 [VLA-4]) so that they can bind to ICAM-1 and VCAM-1, respectively. Not only is the integrin binding of sufficiently high affinity to arrest the CLA-positive T cells, but it also favors the flattening of the lymphocytes in preparation for their extravasation through the endothelial layer. Once extravasated on the abluminal side of the vessel, the T cells are no longer subjected to shear forces from blood flow, and they can respond to chemotactic gradients emanating from the site of injury or infection. If these T cells encounter antigen in tissue, they will become activated. The subsequent release of T-cell cytokines will modify and expand the inflammatory infiltrate.

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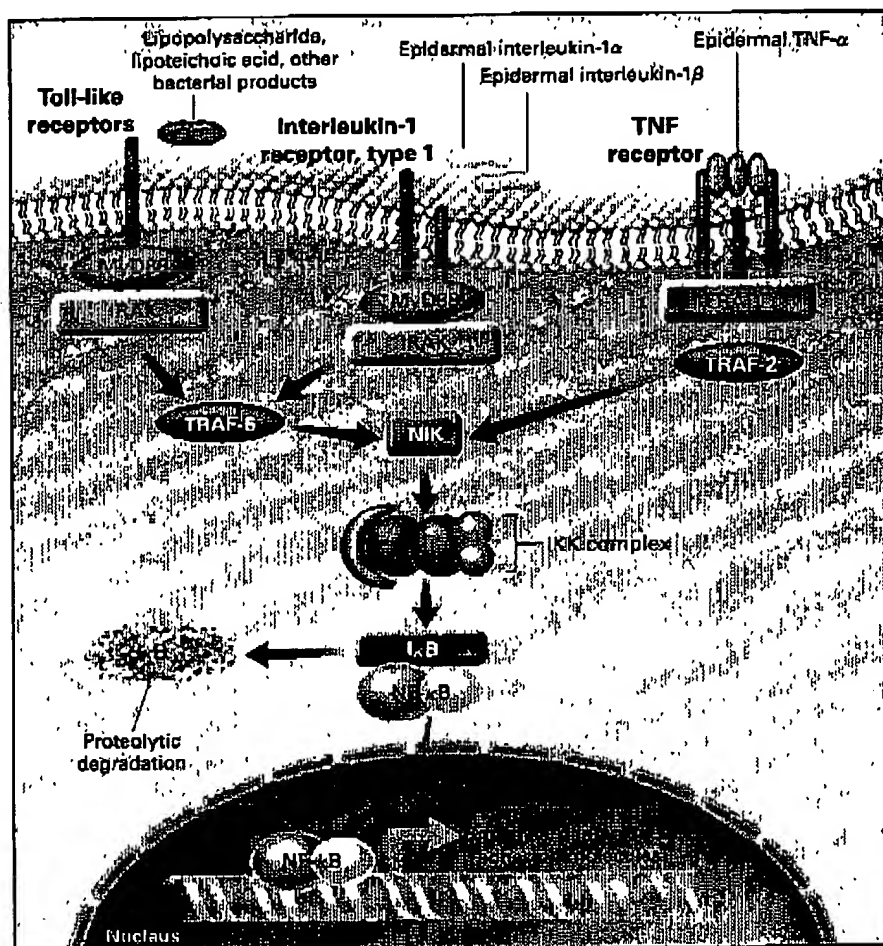


Figure 3. Shared Pathways in Primary Cytokine (Interleukin-1 and Tumor Necrosis Factor α) and Toll-like-Receptor Signaling.

Activation through Toll-like receptors, the interleukin-1 receptor, and the tumor necrosis factor (TNF) receptor all culminate in NF- κ B gene transcription and the production of inflammatory mediators. Toll-like receptors bind microbial products and initiate signaling by recruiting MyD88, an adapter protein also used by the type 1 interleukin-1 receptor after ligand binding. This leads to the recruitment of interleukin-1-receptor-associated kinases (IRAK) 1 and 2, also known as innate immune kinases. TNF-receptor-associated factor 6 (TRAF-6) is recruited to this complex, which then activates NF- κ B-inducing kinase (NIK). NIK is similarly activated by TNF-receptor-associated factor 2 (TRAF-2), which is recruited to the signaling complex of the TNF receptor and its adapter proteins TNF-receptor-associated death domain (TRADD) and receptor-interacting protein (RIP) after ligand binding to the receptor. NIK phosphorylates the IKK complex, which in turn phosphorylates the cytoplasmic complex of I κ B and NF- κ B. This leads to the degradation of I κ B in the cellular proteasome and allows free NF- κ B to migrate into the nucleus. NF- κ B-mediated gene transcription induces the expression of E-selectin, intercellular adhesion molecule 1, and vascular adhesion molecule 1 in cutaneous endothelial cells. It also induces primary cytokine production and chemokine production in keratinocytes, fibroblasts, and other resident skin cells. Collectively, these signals recruit CLA-positive T cells (as well as other leukocytes) from skin.

not by itself require antigen recognition by T cells. For T cells to perform effector functions in skin, however, they must recognize antigen through their antigen receptors. They then become activated, producing effector molecules, including type 1 or type 2 T-cell cytokines. Therefore, only CLA-positive T cells that actually encounter the antigen for which their antigen receptor is specific will be activated during a given episode of cutaneous inflammation. The cutaneous microenvironment favors antigen presentation; antigen-presenting cells are abundant in skin, and blood dendritic cells and monocytes can also be recruited from blood in response to cutaneous inflammation,²⁸ resulting in an expanded pool of these cells. It is the activation of T cells by antigen, and the subsequent release of type 1 and type 2 T-cell cytokines and other effector molecules, that result in clinically apparent, T-cell-mediated skin disease. Type 1 T-cell cytokines induce resident skin cells to produce chemokines that recruit monocytes and additional type 1 T cells. Type 2 T-cell cytokines induce a different set of chemokines that favor the recruitment of eosinophils and type 2 T cells.

Although it facilitates the process dramatically, inflammation may not be an absolute requirement for the extravasation of CLA-positive T cells into the skin. Because cutaneous postcapillary venules express low levels of E-selectin and intercellular adhesion molecule 1 constitutively, activated CLA-positive circulating T cells may not require chemokines to extravasate in the absence of cutaneous inflammation. Alternatively, if low levels of chemokines are constitutively expressed on postcapillary venular endothelium in uninfamed skin,²⁹ even resting CLA-positive T cells may undergo the process of tethering, activation, and adhesion required for extravasation into normal skin. This may represent an additional component of immune surveillance mediated by CLA-positive memory T cells.

What is the fate of the large numbers of CLA-positive T cells that successfully extravasate but do not encounter the antigen for which their antigen receptor is specific during a given episode of inflammation? These cells do not become activated through their antigen receptors, and they appear to leave the skin through afferent lymphatics, traveling to a lymph node and then through efferent lymphatics back to the blood. They then rejoin the circulating population of CLA-positive T cells (Fig. 1) and continue to mediate cutaneous immune surveillance.

T-CELL-MEDIATED SKIN DISEASES

Psoriasis

Psoriasis affects more than 2 percent of the world's people. It is characterized by scaly, red cutaneous plaques that contain inflammatory infiltrates and epidermal hyperproliferation (Fig. 4). The serendipitous observation that treatment with cyclosporine dramati-

cally improved psoriasis²⁹ provided the first strong evidence that the disorder had an immune cause, an idea that had previously been suggested by its association with certain HLA class I haplotypes.³⁰ The role of T cells in this disorder has been demonstrated by the remission of severe psoriasis after treatment with a drug consisting of diphtheria toxin and the receptor-binding domain of interleukin-2 (DAB₃₈₀-interleukin-2), which creates a toxin specific for activated T cells.³¹ Cutaneous T cells in psoriatic lesions express CLA, whereas those found in the joints of patients with psoriatic arthritis do not express CLA.³² Although CD4+ T cells may help initiate the skin lesions, CD8+ T cells that produce type 1 cytokines (interferon- γ) are responsible for the persistence of the lesions.³³⁻³⁵ The role of CD8+ T cells explains the paradox that psoriasis can worsen dramatically even as CD4+ T-cell counts fall in patients with human immunodeficiency virus infection.³⁶ There is increasing support for the idea that psoriasis is an autoimmune disease; however, the antigen or antigens responsible for activating the CD8+ cells in the epidermis are not known.

The development of new psoriatic lesions on injured skin, known as the Koebner phenomenon,³⁷ is consistent with the immune-surveillance paradigm. Normal human epidermis contains preformed interleukin-1 α ,²⁰ which is released from keratinocytes after minor trauma.³¹ CLA-positive T cells are recruited from peripheral blood by interleukin-1 α -initiated inflammation, and CLA-positive T cells whose antigen receptor is specific for the putative psoriatic auto-antigen in skin will be activated *in situ*. The subsequent release of type 1 T-cell cytokines results in further inflammation, the recruitment of additional CLA-positive T cells, and ultimately the development of psoriatic lesions in susceptible persons. The prevalence of psoriasis on the elbows, knees, and other sites of repetitive trauma is consistent with this model. Another potential connection with the innate immune system involves the association of acute exacerbations of established psoriasis with bacterial and fungal infections of skin.³⁸ This interesting clinical observation may have as its basis the activation of Toll-like receptors by infectious microorganisms in skin cells, which induces NF- κ B-mediated inflammation and the recruitment of CLA-positive T cells.

Although activated T cells are necessary for the development and persistence of lesions, psoriasis is difficult to explain solely on the basis of T-cell activation. For example, the activation of CLA-positive T cells that produce type 1 cytokines in the epidermis is probably a common response to environmental antigens in persons in whom psoriasis never develops. Whether this paradox can be explained by the existence of a unique subgroup of cytokines produced by T cells in patients with psoriasis or whether resident skin cells from patients with psoriasis have

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an aberrant response to cytokines or other effector molecules is not known. The clinical heterogeneity of psoriasis and the apparent multigenic pattern of inheritance suggest that a combination of variables are involved in its development.

Therapies for psoriasis in particular, and for T-cell-mediated skin diseases in general, tend to have a remittent effect (inducing long-term remission) or a suppressive effect (improving lesions but with a prompt recurrence when the treatment is discontinued). The differences in remittent and suppressive therapies for psoriasis are correlated with the clinical and histologic features of the disease, such as T-cell apoptosis.³⁹ For example, treatment with ultraviolet B radiation or psoralens plus ultraviolet A radiation (PUVA) greatly reduces the number of activated T cells in the epidermis and dermis of psoriatic skin by inducing T cell apoptosis, often resulting in long-standing remissions.⁴⁰⁻⁴² Systemic treatments with agents such as methotrexate and DAB₃₈₉-interleukin-2 preferentially induce apoptosis of activated T cells, both in blood and in skin.^{31,43} In contrast, treatment with topical corticosteroids or cyclosporine inhibits the production of cytokines by intralesional T cells. Although such suppressive therapies efficiently reduce both inflammation and hyperproliferation of keratinocytes, they rarely reduce the number of lesional T cells to a level below 50 percent of the pretreatment levels.⁴⁴ As a result, psoriasis often recurs soon after the cessation of suppressive therapies. Both remittent and suppressive therapies have toxic effects that may limit their use.

Allergic Contact Dermatitis

Allergic contact dermatitis, also known as contact hypersensitivity, is a T-cell-dependent skin disease with the kinetics of a delayed-type hypersensitivity response⁴⁵ (Fig. 4). This disorder is even more prevalent than psoriasis, and although it is rarely life-threatening, the costs to society of occupation-related allergic contact dermatitis are high.⁴⁶ In this disorder, the offending antigen is introduced epicutaneously through intact skin. The sensitizing antigens are typically unstable reactive molecules that can form complexes with host proteins. In addition, potent contact-sensitizing antigens induce dose-dependent cutaneous irritation that is independent of their antigenicity.⁴⁷ This injury-mediated triggering of the innate immune system may operate through the production of cytokines by resident cells of the epidermis and dermis or through direct activation of the NF- κ B pathway in the endothelium.²² In both cases, endothelial adhesion molecules are expressed and inflammatory chemokines are produced, allowing the recruitment of circulating CLA-positive T cells. These signals also favor the migration of Langerhans' cells bearing contact-sensitizer-modified proteins from the epidermis into draining lymph nodes for presentation to naive T cells.

Within days after the initial cutaneous contact with the sensitizing antigen, newly generated CLA-positive memory T cells specific for this antigen exit the cutaneous lymph nodes and appear in the peripheral blood.⁴⁸ Repetitive exposure to the sensitizing antigen is likely to increase the number of antigen-specific CLA-positive memory T cells circulating in the peripheral blood, until a level is reached that results in allergic contact dermatitis on subsequent exposure. These newly generated CLA-positive T cells extravasate at the site of irritation from the sensitizing antigen, recognize the antigen in situ, and become activated. Their cytokines (and possibly direct cell-mediated injury of keratinocytes) induce the clinical pattern of cutaneous inflammation that is characteristic of allergic contact dermatitis. Subsequent encounters with the contact-sensitizing antigen, even months later, will again lead to the recruitment of CLA-positive T cells from peripheral blood, which now include antigen-specific memory T cells (generated from previous encounters with the contact-sensitizing antigen). T-cell extravasation, followed by antigen-receptor activation and release of T-cell cytokines, leads to the "recall" development of full-fledged clinical allergic contact dermatitis. If the contact-sensitizing antigen is a compound in the workplace that is impossible to avoid or that cannot be identified, the problem may lead to an inability to work in that environment.

Atopic Dermatitis

Atopic dermatitis can be viewed as an exaggerated cutaneous immune response to environmental antigens. Patients with this disorder have a humoral response characterized by IgE antibodies associated with T cells that produce type 2 cytokines (Fig. 4).^{49,50} The antigens that induce such responses are termed allergens, and the allergens frequently responsible for atopic dermatitis are derived from the house-dust mite *Dermatophagoides pteronyssinus*. Atopic dermatitis can be associated with asthma and allergic rhinitis, and there is a strong though incompletely defined genetic component of this disease.

Many lines of evidence suggest that naive, allergen-specific T cells in patients with atopic dermatitis are preferentially induced to develop into CLA-positive T cells that produce type 2 cytokines and migrate to the skin after encountering antigens in skin-draining lymph nodes. CLA-positive CD4⁺ memory T cells specific for such allergens are found in blood from patients with atopic dermatitis but not in blood from normal subjects.⁴⁸ Type 2 T-cell cytokines promote the growth and activation of eosinophils (interleukin-5), a switch in the antibody isotype from IgM to IgE (interleukin-4 and interleukin-13), and a reduction in cell-mediated immunity (interleukin-10).^{20,49} Patients with atopic dermatitis have diminished resistance to cutaneous infections because of this relative cellular immunodeficiency. For example,

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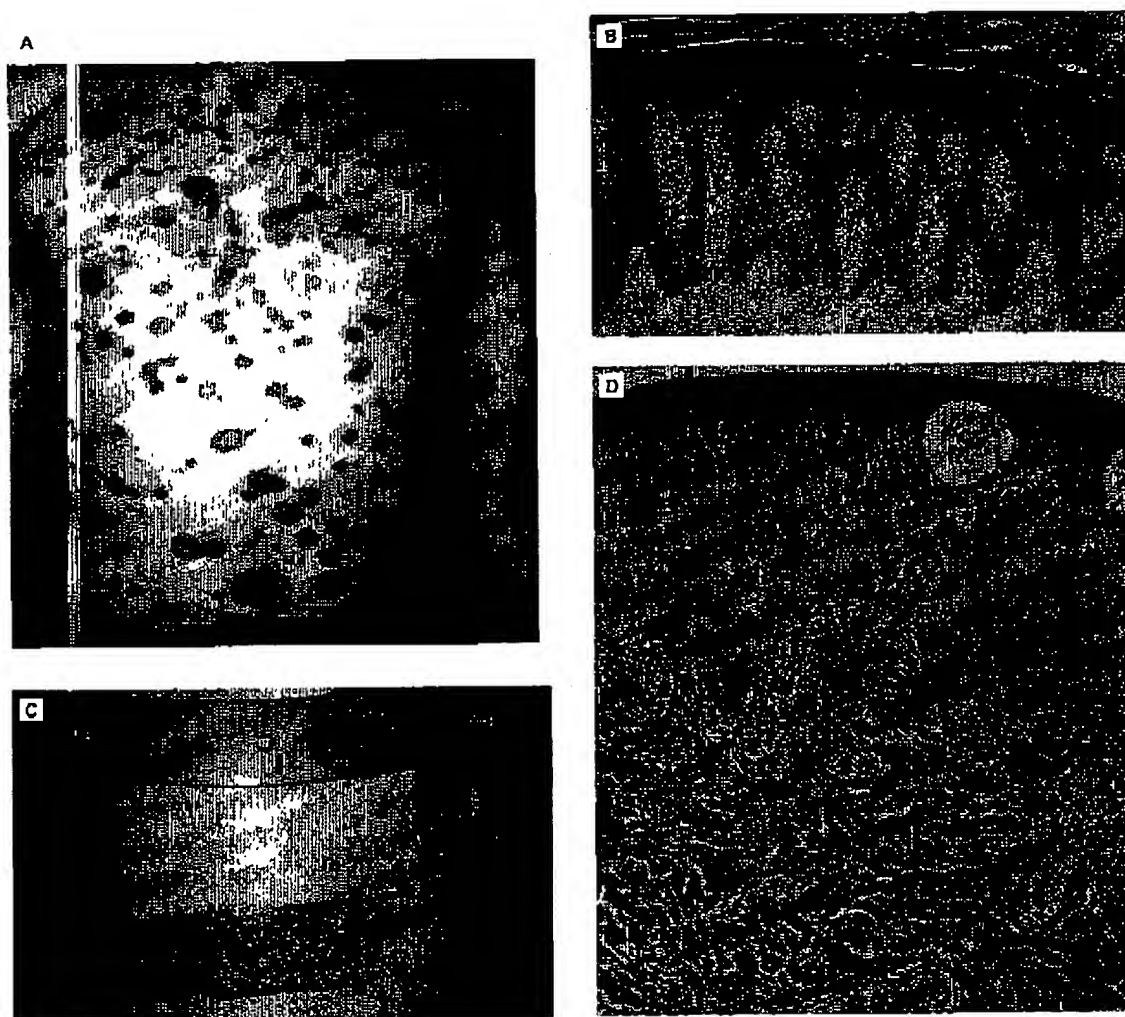


Figure 4. Clinical and Histologic Features of Inflammatory Skin Diseases.

Psoriasis is characterized clinically by scaly erythematous plaques (Panel A) and histologically by epidermal hyperplasia, elongation of dermal papillae, subcorneal neutrophilic pustules, and a dermal and epidermal infiltrate of T cells and monocytes (Panel B; hematoxylin and eosin, $\times 82$). The disorder is mediated largely by CLA-positive, CD8+ T cells with type 1 cytokines (interferon- γ , interleukin-2, and lymphotxin); these cells may be activated by an autoantigen in skin. Psoriasis is a chronic, persistent, often lifelong disease.

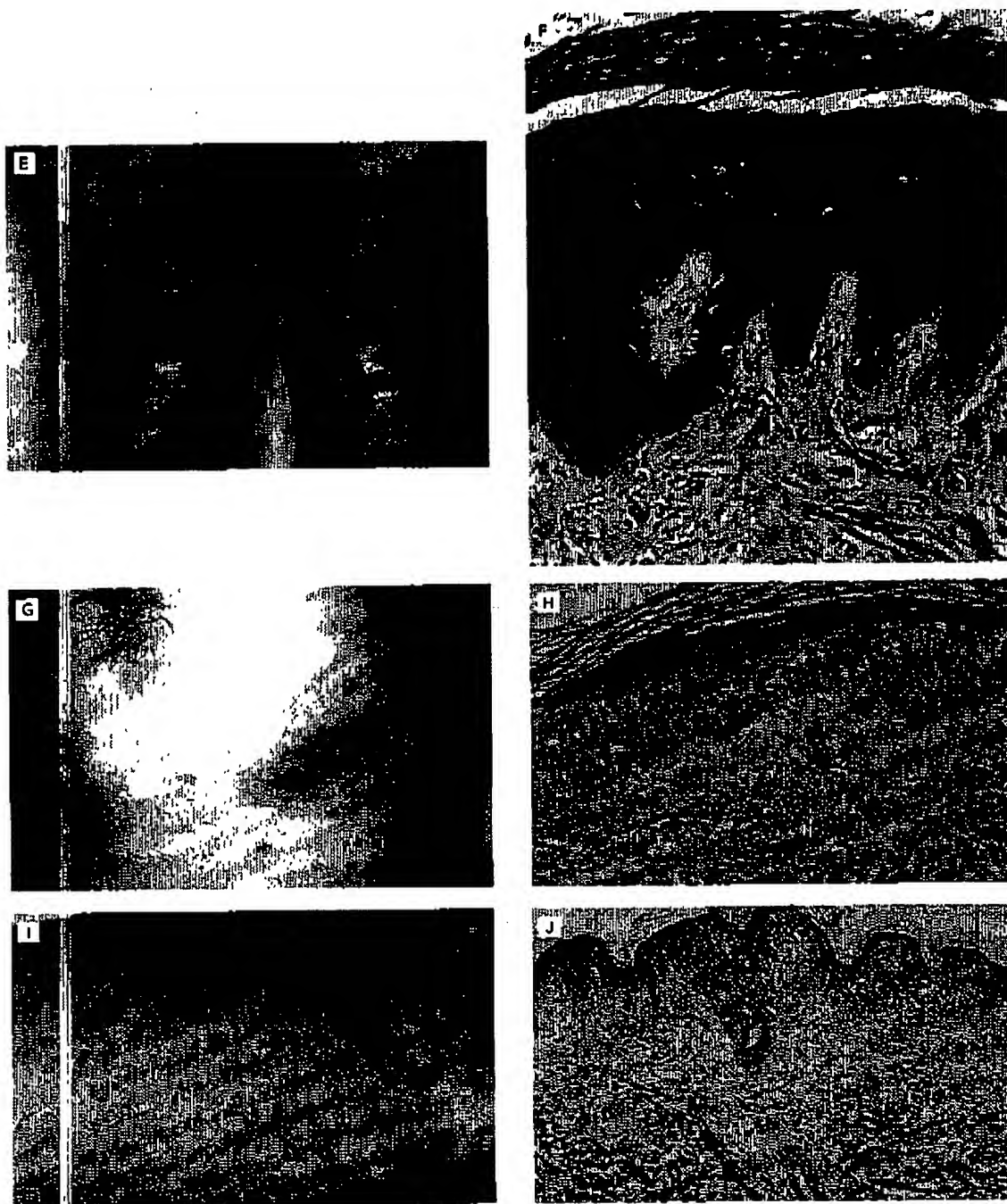
Allergic contact dermatitis is characterized clinically by intense pruritus, erythema, and vesiculation (Panel C) and histologically by spongiosis (intraepidermal edema) and a mononuclear infiltrate (Panel D; hematoxylin and eosin, $\times 87$). It is mediated by CLA-positive, CD8+ effector T cells that recognize contact-sensitizing antigens (small reactive molecules that enter through the epidermis). The activated T cells have a variable cytokine profile (e.g., both type 1 and type 2 cytokines).

Atopic dermatitis is characterized by intense pruritus and erythema and, in its chronic form, by scaling and lichenification (thickening of the epidermis) (Panel E). The characteristic histologic finding is a mononuclear dermal infiltrate in association with epidermal hyperplasia (Panel F; hematoxylin and eosin, $\times 85$). Atopic dermatitis is initiated by CLA-positive, CD4+ T cells with type 2 cytokines (interleukin-4, 5, 10, and 13). T cells that produce type 1 cytokines may be involved in persistent lesions. Environmental allergens, such as proteins from the house-dust mite, *Dermatophagoides pteronyssinus*, trigger the disorder.

Cutaneous T-cell lymphoma is usually manifested clinically as erythematous patches and plaques with minimal scale (mycosis fungoides) (Panel G), though there may be other clinical manifestations (e.g., erythroderma). The transformed T cells are found throughout the dermis and in the epidermis (Panel H; hematoxylin and eosin, $\times 79$), where they may accumulate with Langerhans' cells (Pautrier's microabscesses). Many reactive (nontransformed) CLA-positive T cells are also present in lesions. Mycosis fungoides is a tumor of CLA-positive, CD4+ T cells.

Cutaneous graft-versus-host disease is a complication of allogeneic bone marrow transplantation. The acute form of the disease is

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characterized by a maculopapular exanthem (Panel I), whereas the chronic form may be characterized by marked dermal sclerosis (not shown). Dermal lymphocytic infiltration is associated with characteristic cytopathic changes in keratinocytes (Panel J; hematoxylin and eosin, $\times 63$). CLA-positive T cells producing type 1 cytokines (in acute disease) or type 2 cytokines (in chronic disease) are present in lesions. The disease is caused by the recognition of antigens on host tissue by T cells transferred with the allograft. The photograph in Panel A was provided by Dr. Selim Aractingi, the photograph in Panel C by Dr. Stephan Grabbe, and the photomicrograph in Panel H by Dr. Isabelle Moulouquet.

reactivation of herpes simplex virus infection in such patients can lead to generalized cutaneous disease, requiring systemic antiviral therapy.

A variety of factors may stimulate the inflammation that recruits the T cells that initiate and perpetuate atopic dermatitis.⁴⁹⁻⁵¹ Proteases secreted by mites may cause epidermal injury, leading to the production of primary cytokines and NF- κ B-induced inflammation, and the binding of IgE on mast cells to allergens induces inflammation through the degranulation of the mast cells. Additional triggers may be bacterial activation of Toll-like receptors by cutaneous bacteria and the release of stored interleukin-1 α from skin in response to the trauma induced by scratching or rubbing severely pruritic skin.

Cutaneous Graft-versus-Host Disease

Cutaneous graft-versus-host disease is a common and debilitating complication of allogeneic bone marrow transplantation. This disease is mediated by T cells transferred with the bone marrow allograft (Fig. 4).⁵² The two organs most often affected by graft-versus-host disease — the skin and gastrointestinal tract — are associated with different subgroups of memory T cells that home to these locations. T cells in lesions of cutaneous graft-versus-host disease are positive for CLA, whereas those in the inflamed gastrointestinal tract are negative for CLA but are positive for $\alpha 4\beta 7$ integrin.² A hypothesis currently being tested is that the memory T cells that mediate cutaneous graft-versus-host disease have previously been exposed to antigen in lymph nodes that drain the skin, whereas the memory T cells that mediate gastrointestinal graft-versus-host disease have been exposed to antigen in mesenteric lymph nodes.

Cutaneous T-Cell Lymphoma

Cutaneous T-cell lymphoma, the most common form of T-cell lymphoma in adults, encompasses several discrete diseases that can have markedly different clinical courses.⁵³⁻⁵⁵ The most common variant is mycosis fungoides, which is classified as a low-grade T-cell lymphoma.⁵⁶ Mycosis fungoides is initially manifested as an inflammatory skin disease (Fig. 4), and in early lesions transformed T cells exit the vessels and enter inflamed skin through the CLA-mediated pathway (Fig. 2). Mycosis fungoides is considered to be a lymphoma involving CLA-positive, CD4+ memory T cells that home to skin.

Therapy for mycosis fungoides depends to a large extent on the site at which the malignant T cells are most abundant. In patients with disease limited to the skin, skin-directed therapies such as the administration of psoralens plus ultraviolet A radiation, total-skin electron-beam therapy, topical administration of nitrogen mustard, and ultraviolet B radiation often induce long-lasting remissions.⁵⁵ The apparent paradox of a systemic lymphoma (e.g., blood

involvement in an early stage of the disease, as determined by molecular analysis⁵⁶) that can be put into durable remission by therapies that do not extend beyond the skin probably reflects the stringent homing patterns of these cells. If most continuously recirculating mycosis fungoides cells reside in skin, with very few such cells in blood or lymph nodes, then repeated courses of skin-directed therapy for a period of weeks to months may eliminate the vast majority of the cells. This principle reflects the efficacy of skin-directed therapy in immunologically mediated, nonmalignant skin disease. In advanced stages of mycosis fungoides, the T cells have lost their strict dependence on skin, and systemic therapy is required.⁵⁵ Systemic therapy, which includes interferon α , retinoids, DAB₃₉-interleukin-2, and photopheresis, is often used in conjunction with skin-directed therapy. Combination chemotherapy may be palliative, but curative regimens for advanced disease have not yet been developed.

CONCLUSIONS

The ability to respond rapidly to a pathogen after the first encounter with it is the hallmark of acquired immunity and immunologic memory. The rapid, site-specific accumulation of CLA-positive T cells after cutaneous injury meets this criterion. CLA-positive T cells extravasate in response to inflammatory signals from skin; thus, the immune system regards insults to the skin as potential infectious challenges until proved otherwise. T-cell-mediated skin diseases such as the ones we have discussed represent a subversion of this highly adaptive process. Although each of these disorders can be viewed as an example of inappropriate cutaneous immune surveillance, their clinical manifestations and courses are determined by several factors: the functional phenotype and cytokine profile of the antigen-specific T cell, the type of antigen (e.g., pathogen, autoantigen, or contact-sensitizing antigen), and the genetic background of the person. This last variable, which is the most complex and the least well defined, is the focus of much of the current research on T-cell-mediated inflammatory skin diseases.

An increased understanding of the mechanisms of cutaneous immune surveillance will almost certainly provide important insights into diseases at other epithelial interfaces with the environment, in view of the fact that microvascular beds are morphologically and functionally different in different parts of the body. This is particularly true of the high endothelial venules in lymphoid tissue,⁵⁷ which favor the homing of naive lymphocytes⁸; cutaneous microvascular endothelial cells with prolonged expression of E-selectin¹⁷; and endothelial cells of the lamina propria in the gastrointestinal tract, which express an adhesion molecule that favors the homing of $\alpha 4\beta 7$ -positive memory T cells.^{58,59} Preferential expression of differ-

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ent chemokines in these tissues may provide for further specificity of T-cell homing.¹⁹

What we now know about the movement and function of naive and memory T cells suggests that from the perspective of the host defense against environmental challenges, regional immune responses have a central role in the body's response to infectious challenge — the *raison d'être* of the immune system. Therapies directed at the movement patterns of T lymphocytes — either positively or negatively — are likely to be important elements in the future treatment of inflammatory skin diseases.

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ORIGINAL PAPER

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The action of a novel vitamin D₃ analogue, OCT, on immunomodulatory function of keratinocytes and lymphocytes

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Abstract Topical vitamin D₃ has relatively recently been introduced for the treatment of psoriasis. Synthetic vitamin D₃ analogues with a high potential for inducing differentiation of cells, but with a low hypercalcemic effect have recently been developed. One such synthetic analogue of 1,25-dihydroxyvitamin D₃ (calcitriol), 22-oxacalcitriol (OCT), is a novel agent for the topical treatment of psoriasis. The activity of OCT *in vitro* was investigated and compared with that of a series of vitamin D₃ analogues as to their ability to inhibit murine T lymphocyte proliferation stimulated by con-A, to suppress IL-6 and IL-8 production by keratinocytes stimulated with IL-1 α and TNF α , and to inhibit AP-1- and NF κ B-dependent reporter gene expression. OCT inhibited the proliferation of lymphocytes and suppressed IL-8 and IL-6 production by keratinocytes to the same extent as the other vitamin D₃ analogues. It also inhibited AP-1- and NF κ B-controlled luciferase activity to the same extent as the other vitamin D₃ analogues, which demonstrates its mechanism of action in the suppression of inflammatory processes.

Key words Vitamin D₃ · OCT · Cytokines · Nuclear factors

Introduction

Psoriasis is characterized by hyperproliferation and incomplete terminal differentiation of the epidermis, vascular changes involving elongation and dilatation of capillaries in the papillary dermis, and migration of activated neutrophils and T lymphocytes into the dermis and the epidermis [8]. The pathogenesis of psoriasis is not fully understood, but many abnormalities in psoriasis are well documented including abnormal infiltrating lymphocytes, neutrophils, circulating leukocytes and cytokines, and epidermal keratinocytes [10, 17, 30]. Psoriatic keratinocytes produce a much higher amount of TGF α , IL-6, and IL-8 than normal keratinocytes, and these cytokines are suspected to be the cause of hyperproliferation and neutrophil infiltration of the psoriatic epidermis [10, 17].

No curative agent is yet known for psoriasis. Topically applied steroids, and systemically administered cyclosporin, retinoids, and methotrexate are the major therapeutic modalities, but have only a temporary effect. The biologically active metabolite of vitamin D₃, the secosteroid hormone 1 α ,25-dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃, calcitriol), exerts profound effects on cellular differentiation and proliferation and immunomodulation, and is one of the effective agents for treating psoriasis [12]. The effect of topical vitamin D₃ appears slowly but its side effects are much more favorable than those of topical steroids.

Vitamin D₃ is produced from 7-dehydrocholesterol in the skin by the action of sunlight, and is transported via the blood to the liver where it is converted into 25(OH)D₃. A subsequent reaction produces the active metabolite of vitamin D₃, 1 α ,25(OH)₂D₃ (calcitriol), in the kidneys [9]. Receptors for calcitriol have been found in epidermal keratinocytes, dermal fibroblasts, endothelial cells, and activated T lymphocytes [7].

In cultured human keratinocytes, calcitriol inhibits cell proliferation and induces terminal differentiation [26], and thus brings hyperproliferative and activated keratinocytes to a stable state, which could result in clinical im-

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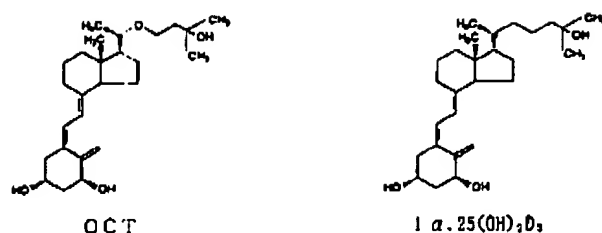


Fig. 1 Molecular structure of OCT and calcitriol

provement in psoriasis. Calcitriol also inhibits immunological responses by attenuating signal transduction cascades elicited by cytokines and growth factors. As glucocorticoids and vitamin A, vitamin D₃ metabolites also act to suppress the expression of activated IL-2 expression and GM-CSF at the level of transcription [32]. Vitamin D₃ is also known to induce IL-10 receptor expression [17], which may also help in the resolution of psoriasis. Topical treatment of psoriasis with calcitriol has produced successful results [12]. The clinical use of calcitriol, however, is limited because of its potent effect on systemic calcium metabolism. Hypercalcemia can be induced by oral doses of calcitriol higher than a few micrograms daily, and transepidermal absorption occurs with topical application. There have, therefore, been attempts to synthesize new vitamin D₃ analogues with a lower risk of inducing the classic side effects associated with vitamin D₃, i.e. hypercalciuria and hypercalcemia [9].

Vitamin D₃ analogues with selective activity which retain cell-differentiating activity but have very little hypercalcemic activity have recently been developed. One such analogue is 22-oxacalcitriol (OCT) in which the carbon atom at position 22 of calcitriol is replaced by oxygen (Fig. 1). OCT has strong differentiation-inducing activity, but low bone-resolving activity [1]. This separation of activities is probably a result of differences in their physicochemical properties, i.e. (1) the length of the side-chain (between C20 and C25) of OCT (4.96Å) is shorter than that of calcitriol (5.15Å), (2) the rotation of the side-chain of OCT is more strictly limited than that of calcitriol owing to the presence of the oxygen atom at position 22 in OCT, and (3) OCT is more polar than calcitriol [1]. Okano et al. [21] have reported that OCT has a very low affinity for serum vitamin D-binding protein (DBP). Furthermore, as predicted by its low affinity for DBP, OCT is rapidly cleared *in vivo* and more accessible to target cells in culture [6], which is thought to be the reason for the separation of its potential for differentiation and its hypercalcemic effect.

This new synthetic vitamin D₃ analogue has been investigated in a clinical study in which, when given once daily at a concentration of 25 µg/g, it produced more favorable results than calcipotriol at 50 µg/g, one of the newly synthesized vitamin D₃ analogues which is already in clinical use (Barker et al., submitted for publication).

We present here the results of a study of the modulatory effects of several vitamin D₃ analogues, including

OCT, on the immunological aspects of psoriasis such as cytokine production by keratinocytes and lymphocyte proliferation. IL-1α, TGFα, and TNFα are the major factors capable of inducing IL-6 and IL-8 production by keratinocytes [19]. Using these cytokines as stimulators of keratinocytes, we tested the inhibitory effect of OCT on the production of IL-6 and IL-8. Furthermore, in order to investigate the mechanism of action of OCT and other vitamin D₃ analogues, we studied their inhibitory effect on the transcriptional activities of AP-1 and NFκB. This study is the first systematic examination of the *in vitro* effects of these vitamin D₃ analogues.

Materials and methods

Lymphocyte preparation and [³H]thymidine incorporation

C3H/HeN Crj mice (15 weeks old, male) were purchased from Japan Charles River (Yokohama, Japan) and the spleens were used for lymphocyte preparation following the Principles of Laboratory Animal Care (NIH publication no. 85-23).

Lymphocytes were obtained from mice spleens by density gradient centrifugation with lymphocyte-M, seeded in 96-well plates at 5×10^5 cells/well with 4 µg/ml of succinylated concanavalin A (con-A) in RPMI-1640 containing 5% fetal calf serum (FCS) for 5 days together with the vitamin D₃ analogues added to the medium at 10^{-12} to 10^{-6} M. After 2 days of culture, the medium was changed and 3 days later, cells were pulsed with 1 µCi/ml [³H]thymidine. After a further 4-h incubation the incorporation of TdR into the cells was measured with a plate.

Cell culture

Keratinocytes were expanded from normal human skin biopsy specimens in the presence of X-ray-irradiated 3T3 cells, according to the method of Green [25]. Fibroblasts were removed by incubating them with 0.05% EDTA before trypsinization. The next passage of cells consisting only of keratinocytes were plated into 96-well plates coated with type IV collagen for the IL-6 experiment. In other cases, keratinocytes were passaged and grown in keratinocyte serum-free medium, and were plated into 24-well plates. When cells had proliferated to confluence, IL-1α was added to a final concentration of 50 U/ml to the medium. At the same time, the vitamin D₃ analogues OCT, TV-02 (tacalcitol), MC-903 (calcipotriol) and calcitriol were added at concentrations in the range 10^{-11} to 10^{-6} M with EtOH at a final concentration of 0.1%. After the times indicated below, the supernatants were collected and the concentrations of IL-6 and IL-8 measured.

Measurement of IL-6 and IL-8

For measurement of cytokines, the supernatants from keratinocytes were collected after 3, 6, 12, 24, and 48 h incubation with or without IL-1α, TNFα or TGFα with the wide range of vitamin D₃ analogues indicated above. The concentrations of IL-6 and IL-8 were determined using an IL-6 ELISA kit and an IL-8 ELISA kit. The optical density was measured using a microplate reader (Bio-rad, Richmond, Calif.).

Construction of reporter genes and vitamin D receptor expression vector

Two kinds of reporter genes were constructed using luciferase which binds to the IL-2 promoter (-72 to +42) via the TATA box. One had five repeats of the AP-1-binding sequence 'ATGAGT-

CAG' derived from the promoter of the collagenase gene (AP-1-Luc). The other had four repeats of the NF κ B-binding sequence 'CAGAGGGGACTTTTCCGAGA' derived from the promoter of the immunoglobulin light κ gene (NF κ B-Luc). For vitamin D receptor (VDR) expression, rat VDR cDNA was inserted into the pSG5 expression vector (Stratagene, Heidelberg, Germany).

Transfection and luciferase assay

The VDR expression vector (1 μ g) and one of the reporter genes (1 μ g) were cotransfected into Jurkat (S3C4) cells ($3-5 \times 10^6$) using lipofectamine on day 1. On the following day (day 2), the treated cells were plated onto 96-well black plates for luminescence measurement at a density of 2×10^5 cells/well. The desired concentrations of vitamin D₃ analogues (10^{-6} – 10^{-11} M) were added to each well, followed by stimulants for AP-1 and NF κ B, which consisted of 10 ng/ml PMA and 1 ng/ml ionomycin. After 8 to 12 h incubation in an incubator with an atmosphere enriched in CO₂, the supernatants were removed and the cells were lysed with a lysis buffer at 20 μ l/well (Promega, Madison, Wis.).

Luciferase assay reagent (Promega) was added to the lysate at 100 μ l/well and the luciferase activities were measured using a luminometer for 5 s/well.

Statistical analysis

Data are expressed as means \pm standard deviations. For each parameter, mean values were compared between the groups using Student's *t*-test or Dunnett's multiple comparison test as indicated in the results, and dose-dependency was analyzed with Jonckheere's test. *P*-values of 0.05 or less were considered significant.

The three vitamin D₃ analogues, OCT, TV-02, and MC-903 were prepared according to previously reported methods [16, 18, 20, 28]. Calcitriol was purchased from the Duphar Company (CP Weesp, The Netherlands). Succinylated con-A was purchased from Vector Laboratories (Burlingame, Calif.). Lymphocyte-M was purchased from Cedarlane (Montreal, Canada). RPMI-1640 medium was purchased from GIBCO (Grand Island, N.Y.). FCS was purchased from HyClone (Logan, Utah). [³H]Thymidine was purchased from Amersham (Buckinghamshire, UK). Type IV collagen was purchased from Nitta Gelatin (Tokyo, Japan). DMBM(H) (Dulbecco's modified Eagle's medium containing high glucose) was purchased from GIBCO. Keratinocyte serum-free medium was purchased from Krabou (Osaka, Japan). Human recombinant IL-1 α (10,000 U/ml) was purchased from Boehringer Mannheim (Mannheim, Germany), and TNF α from R&D Systems (Minneapolis, Minn.). The IL-6 ELISA kit was purchased from Amersham, and the IL-8-ELISA kit from R&D Systems. Lipofectamine was obtained from GIBCO. PMA and ionomycin were purchased from Sigma (St. Louis, Mo.).

Results

Inhibition of con-A-induced mouse lymphocyte proliferation

We investigated first the antiproliferative activity of a series of vitamin D₃ analogues against con-A-stimulated mouse lymphocytes to assess the immunoregulatory effect of vitamin D₃. The results are shown in Fig. 2.

The vitamin D₃ analogues inhibited con-A-induced proliferation of mouse lymphocytes in a dose-dependent manner, and the antiproliferative effect of OCT was similar to that of the other vitamin D₃ analogues TV-02, MC-903 and calcitriol (*P* = 0.2267). OCT and the other analogues at doses of 10^{-6} M and 10^{-7} M inhibited the con-A-

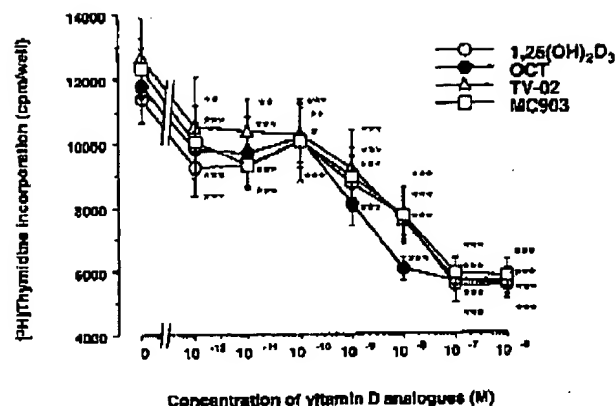


Fig. 2 Proliferation of mouse lymphocytes, induced by concanavalin A, is inhibited by vitamin D analogues. Lymphocytes were stimulated with 4 mg/ml of succinylated concanavalin A, and vitamin D₃ analogues were added at 10^{-12} to 10^{-6} M to the medium. [³H]Thymidine incorporation was measured after 3 days of culture. The results are expressed as the means \pm SD (*n* = 6). OCT, TV-02, MC-903 and 1,25(OH)₂D₃ inhibited lymphocyte proliferation in a dose-dependent manner (*P* < 0.0001, Jonckheere's test). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, vs each control, Dunnett's multiple comparison test

induced proliferation of lymphocytes to almost half that of cells without vitamin D₃. These data were analyzed using Dunnett's multiple comparison test, which showed significant inhibition at concentrations of 10^{-12} to 10^{-6} M of OCT, TV-02, MC-903 and calcitriol (*P* < 0.001, except *P* < 0.01 for 10^{-10} M OCT, 10^{-12} and 10^{-11} M TV-02 and *P* < 0.05 for 10^{-10} M calcitriol).

Inhibition of IL-6 and IL-8 production by human keratinocytes

In order to define the effect of vitamin D₃ analogues on the inhibition of IL-8 production by keratinocytes, supernatants from keratinocytes treated with TNF α , TGF α , and IL-1 α with or without OCT, MC-903 and calcitriol were harvested after 24 h of incubation, which produced the maximal inhibition of IL-8 by keratinocytes incubated with OCT, MC-903 or calcitriol (data not shown).

Figure 3 shows that the vitamin D₃ analogues suppressed IL-8 production by keratinocytes stimulated with TGF α , TNF α and IL-1 α almost to the same extent.

We investigated whether vitamin D₃ analogues suppressed IL-6 production by IL-1 α -stimulated keratinocytes using a wide range of vitamin D₃ concentrations from 10^{-11} M to 10^{-6} M. Vitamin D₃ inhibited IL-6 production in a dose-dependent manner with the strongest inhibition at a concentration of 10^{-6} M. We also compared the dose-dependent curves of each analogue. OCT, TV-02, MC-903 and calcitriol similarly inhibited IL-6 production by human keratinocytes induced by IL-1 α , as shown in Fig. 4. Analysis by Dunnett's multiple comparison test showed the inhibition of IL-6 production by OCT,

Fig. 3 Vitamin D₃ analogues suppress TGF α , TNF α and IL-1 α -induced IL-8 production by keratinocytes. Keratinocytes were incubated with TGF α (10 ng/ml), TNF α (10 ng/ml), or IL-1 α (5 ng/ml), together with or without 10⁻⁶ M of OCT, MC-903, 1 α ,25(OH)₂D₃, or TV-02. The IL-8 concentration of the supernatant was determined by ELISA. The figure shows the inhibition rates, which are the ratios of the concentration in the supernatant with vitamin D₃ analogue to that without vitamin D₃ analogue. These vitamin D₃ analogues significantly inhibited TNF α , TGF α and IL-1 α -stimulated IL-8 production by keratinocytes ($n = 6-8$, ** $P < 0.01$, *** $P < 0.001$, compared to the control without vitamin D analogue, Student's t -test)

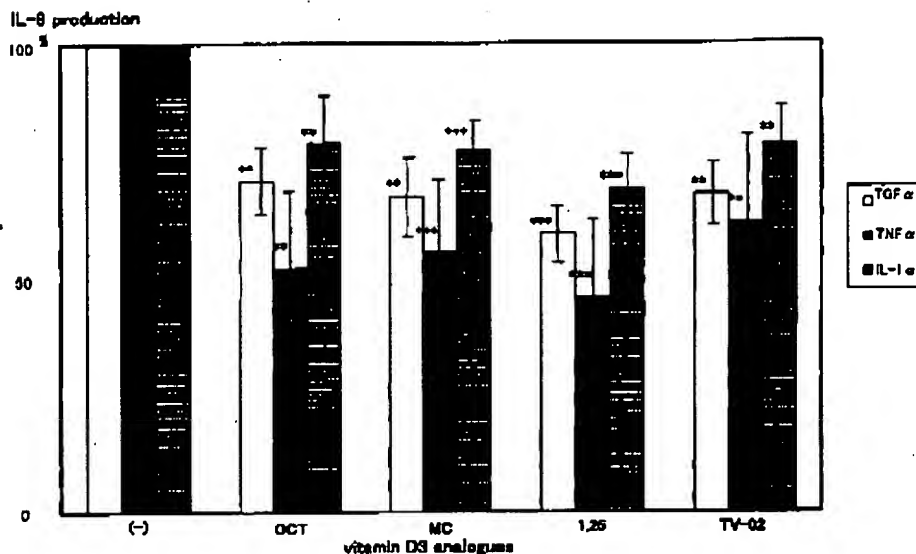
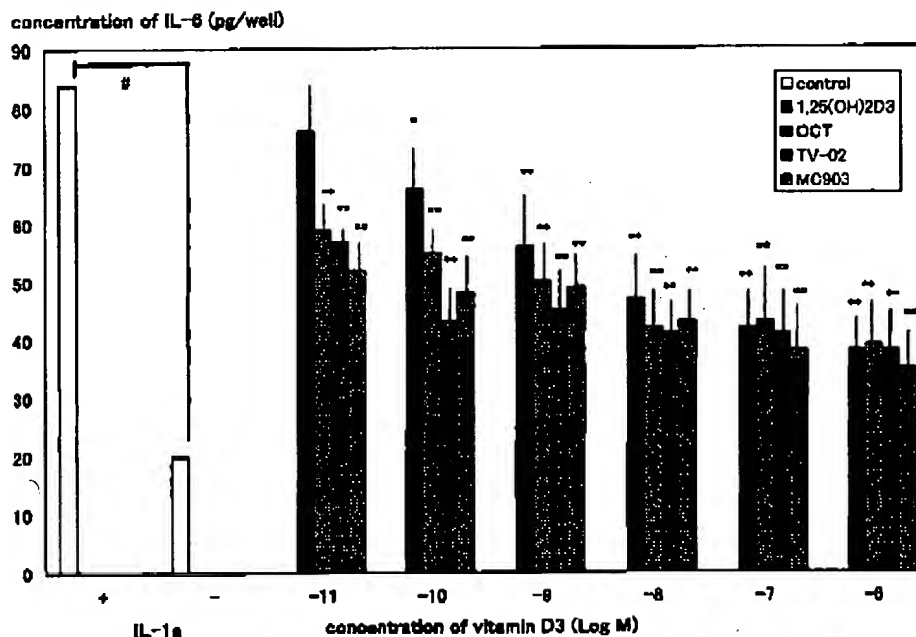


Fig. 4 Vitamin D₃ analogues inhibit production of IL-6 by keratinocytes in a dose-dependent manner. Keratinocytes were incubated with IL-1 α (5 ng/ml), together with a range of concentrations of 1 α ,25(OH)₂D₃, OCT, TV-02, and MC-903. The supernatant from each well was collected after 24 h, and the concentration of IL-6 was determined by ELISA. IL-1 α caused a significant increase in IL-6 production by keratinocytes ($P < 0.0001$, Student's t -test). OCT, TV-02, MC-903 and 1 α ,25(OH)₂D₃ inhibited IL-1 α -stimulated IL-6 production by keratinocytes in a dose-dependent manner ($P < 0.0001$, Jonckheere's test). ** $P < 0.01$, *** $P < 0.0001$, vs control (IL-1 α (+)), Dunnett's multiple comparison test; $n = 6$



TV-02 and MC-903 to be significant at every concentration tested ($P < 0.001$). Calcitriol significantly inhibited IL-6 production at a concentration of 10⁻¹⁰ M ($P < 0.01$) and at concentrations in the range 10⁻⁹–10⁻⁶ M ($P < 0.001$), but not at a concentration of 10⁻¹¹ M.

We also tested the inhibitory effects of some vitamin D₃ analogues on TNF α -stimulated keratinocytes. Figure 5 shows that calcitriol and OCT suppressed TNF α -stimulated IL-6 production by keratinocytes to a similar extent in a dose-dependent manner.

These results suggest that vitamin D₃ works in psoriatic lesions partly through suppressing IL-6 and IL-8 pro-

duction by keratinocytes, and that the vitamin D₃ analogues tested were almost equally efficient in the suppression of IL-6 and IL-8 production.

Inhibition of AP-1- and NF κ B-dependent transcription

IL-1 α , TNF α and TGF α induce IL-6 and IL-8 partly through the nuclear factor NF κ B and/or AP-1 [19, 24]. The VDR, on binding to its ligand, binds to AP-1 with a cofactor and suppresses its activity [11]. We made constructs, one of which consisted of five AP-1 sequences lo-

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Fig. 5 Vitamin D₃ analogues inhibit TNF α -stimulated IL-6 production by keratinocytes. Keratinocytes were incubated with or without TNF α (10 ng/ml), and with or without various concentrations of 1 α ,25-(OH)₂D₃ and OCT. The supernatant from each well was collected after 24 h and the concentration of IL-6 was determined by ELISA. The ratios of the concentration with vitamin D₃ analogues to that without vitamin D₃ analogues was taken after normalization for the number of cells in each well ($n = 2$)

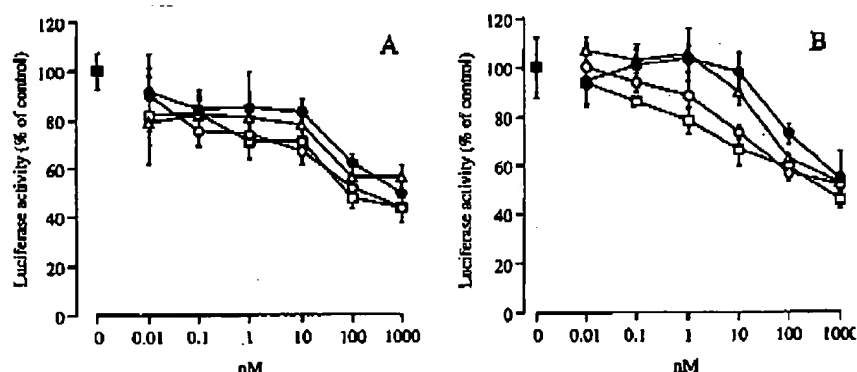
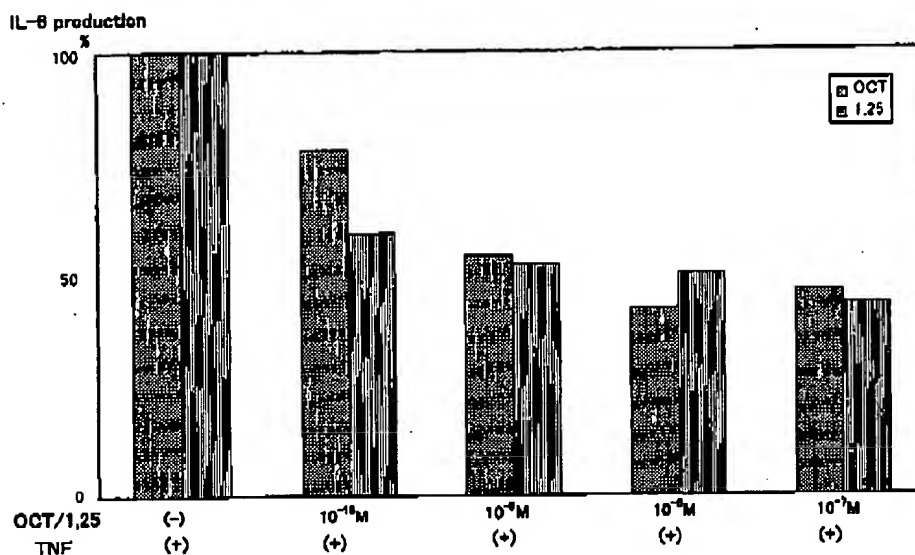


Fig. 6A, B Vitamin D₃ analogues inhibit AP-1- and NF κ B-regulated reporter gene expression in a dose-dependent manner. Either the AP-1 or NF κ B sequences, located upstream of the luciferase gene, were transfected with the VDR expression vector into Jurkat cells. The figure shows the dose-dependent inhibitory effects of vitamin D₃ analogues on the transcriptional activities of AP-1 (A) and NF κ B (B) induced by PMA and ionomycin. Luciferase activity is shown as percentage of the control. These results are representative of six experiments. Each experiment was performed in three to six cultures (● 1,25(OH)₂D₃, ○ OCT, △ TV-02, □ MC-903)

cated in tandem upstream of the luciferase gene as a reporter, and the other consisting of four NF κ B sequences also placed in tandem upstream of the luciferase reporter gene. By transfecting Jurkat cells with each of these vectors together with the VDR expression vector, and treating them with various vitamin D₃ analogues, we found that vitamin D₃ suppressed not only AP-1-Luc activity, but also NF κ B-Luc activity. Vitamin D₃ suppressed AP-1-Luc and NF κ B-Luc activities to 40–50% (Fig. 6), which is relevant to the effect of vitamin D₃ on the production of IL-6 and IL-8 measured by ELISA.

OCT suppressed AP-1-Luc and NF κ B-Luc activity to the same extent as the other vitamin D₃ analogues tested, which is also relevant to the ELISA experiment.

Discussion

Topical vitamin D₃ application is a relatively new method for treating psoriasis [27], but its mechanism of action is not fully understood. Our results support the idea that part of the mechanism of action of vitamin D₃ analogues is on the immunological aspect of psoriasis.

Recent evidence indicates that vitamin D₃ metabolites and analogues also modulate lymphocyte growth and functions. Although lymphocytes require prior activation with antigens or mitogens before expressing VDRs [5, 23], lymphocytes in psoriasis are already activated by various stimuli including IL-1 α , TNF α , IFN γ and IL-2 to express VDR [15]. Our results indicating that vitamin D₃ analogues, including OCT, inhibit con-A-stimulated lymphocyte proliferation dose-dependently could partly explain the clinical effect of vitamin D₃ analogues in reducing infiltrating cells in psoriatic plaques, and the subsequent improvement in psoriatic lesions [14, 33].

Keratinocytes, as well as lymphocytes, are major sources of cytokines and growth factors in psoriasis [15, 22]. We demonstrated that vitamin D₃ not only affected lymphocyte proliferation, but also IL-8 and IL-6 production

by keratinocytes. We examined the effect of vitamin D₃ analogues on the production of IL-6 and IL-8 from stimulated keratinocytes and showed that they inhibit cytokine production in a dose-dependent manner. Although Bikle et al. have reported that under serum-free conditions OCT inhibits keratinocyte proliferation and promotes of differentiation less potently than calcitriol owing to its decreased cellular uptake and enhanced catabolism [6], its inhibitory effect on IL-6 and IL-8 production seemed to be similar to that of other analogues in our study. This inhibition could be due to the inhibition of secretion from keratinocytes, or the inhibition of transcription in keratinocytes. Although we cannot be sure which of these processes was taking place in our experiments, IL-8 production from keratinocytes is reported to be largely regulated at the transcriptional level [19].

AP-1 and NF κ B are transcription factors which are downstream of the signal transduction cascades of the IL-1 receptor, TNF receptor and other cytokine receptors [3, 4], which are involved in the upregulation of IL-8 and IL-6 [19, 24, 31]. The inhibition of cytokine production induced with IL-1 α and TNF α by vitamin D₃ analogues could be due either to inhibition of these transcription factors, or to inhibition of promoter activity through the VDR response element (VDRE).

An inhibitory effect of calcitriol on AP-1 transcriptional activity via the VDR complex has recently been discovered [2]. Very recently, NF κ B binding to its consensus sequence has been found to be suppressed by calcitriol and its receptor complex [11]. Inflammatory cytokines such as IL-6 and IL-8 are upregulated by TNF α and IL-1 α through transcription factors such as C/EBP, NF κ B and/or AP-1 [11, 19]. VDR, upon binding to its ligand, has recently been reported to suppress IL-8 production by affecting NF κ B, and not by directly binding to its consensus sequence on the IL-8 promoter [19]. Our results indicate that OCT has an inhibitory effect on AP-1- and NF κ B-controlled transcriptional activities similar to the effect of other vitamin D₃ analogues. This indicates that vitamin D₃ analogues/VDR complex may make a complex with NF κ B as well as AP-1. This should be further examined using immunoprecipitation assays. Vitamin D₃ suppressed AP-1-Luc and NF κ B-Luc activities to 40–50%, which is relevant to the effect of vitamin D₃ on the production of IL-6 and IL-8 measured by ELISA. Although it may be too simplistic to apply the results in Jurkat cells to keratinocytes, because signal transduction cascades in different cell types can be different, and consequently the nuclear factors used can be different, we speculate that vitamin D₃ inhibits IL-6 and IL-8 production induced by IL-1 α and TNF α through the downregulation of AP-1 and NF κ B.

These inhibitory actions have profound effects on immunological responses, because numerous immunoregulatory genes are regulated through AP-1 and NF κ B nuclear factors, including cytokines such as IL-1, -2, -3, -4, -5, -6, -8, -10 and -13 [7, 21, 30], G-CSF, GM-CSF, TNF α , IFN γ , MCP-1 and PDGF, adhesion molecules such as E-selectin, ICAM-1, VCAM-1, ELAM-1, TCR, MHC-

I and IL-2R α , and enzymes such as iNOS, COX-2, c-myc, MMP-1 and MMP-2 [3, 4]. This leads us to speculate that by inhibiting AP-1- and NF κ B-regulated gene expression, vitamin D₃ suppresses the expression of those genes having profound effects on immunological responses. This could also explain the effectiveness of vitamin D₃ analogues in controlling the clinical course of psoriasis.

As shown above, OCT and the other vitamin D₃ analogues have common immunomodulatory functions, which may have therapeutic effects on psoriasis. In this study, OCT showed an effect almost equal to that of the other vitamin D₃ analogues. Although keratinocyte differentiation and proliferation were not assessed in this study, we have compared OCT with other vitamin D₃ analogues as to its effect on keratinocyte differentiation measured by involucrin mRNA synthesis, and proliferation, and found that OCT was more effective than the other analogues tested (Nakagawa et al., unpublished data). Considering the fact that OCT, in serum-containing medium, has a stronger effect than other vitamin D₃ analogues because of its lower affinity to DBP and faster catabolism, OCT may work more effectively than other vitamin D₃ analogues *in vivo*. This further supports the results of the clinical trial, in which 25 mg/g OCT was more effective than 50 mg/g calcipotriol. This suggests that OCT may be useful in clinically because it has a lower hypercalcemic effect and a stronger antipsoriatic effect.

This study gives the basic immunological background of OCT and provides a step towards understanding its effectiveness in clinical use, and is the first systematic comparison of OCT and various other vitamin D₃ analogues.

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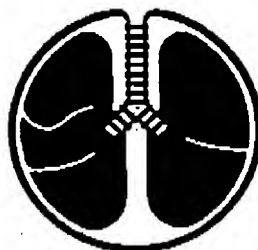
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THE LUNG

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cytes to induce their respective inflammatory functions. However, *in vitro* studies do not always correctly predict *in vivo* results. LTB₄, known to cause neutrophil degranulation and superoxide production *in vitro*, induced the expected neutrophil accumulation when instilled into the lungs of normal volunteers, but the anticipated increases in protein and markers of neutrophil activation in bronchoalveolar lavage fluid did not occur (109,110).

Intercellular adhesion molecule-1 (ICAM-1) is a ligand for the CD11/CD18 leukocyte integrins expressed on the surface of neutrophils, monocytes, lymphocytes, and eosinophils (111,112). Cell-cell contact mediated by leukocyte integrin-ICAM-1 interaction is thought to be important in inflammatory cell-induced target recognition and cytotoxicity (113). In addition, interaction of inflammatory cells with adhesion molecules such as ICAM-1 might allow retention of inflammatory cells in the airway by providing a foothold for their attachment. Cytokines such as TNF, IL-1, and γ -IFN may again play a prominent role in modulating an inflammatory response by enhancing ICAM-1 expression on epithelial cells (114).

Airway epithelial cells are capable of expressing major histocompatibility complex (MHC) antigens (115-117). Expression of MHC antigens allows cells to interact directly with T lymphocytes and makes them potential targets for cytotoxic T cells and candidates for antigen presentation to helper T cells. The expression of MHC class II antigens (also known as human lymphocyte antigens [HLA]-DR, -DQ, -DP) is relatively low in normal airway epithelium, but can be increased by cytokines. The most potent stimulator for MHC class II expression appears to be γ -IFN (109,110), but TNF α may potentiate γ -IFN effects (118). Bronchial epithelial cells that express MHC class II antigens are capable of stimulating allogenic lymphocyte reactions (117). In addition, the capacity of airway epithelial cells to express MHC class II antigens raises the possibility that the epithelial cells are capable of directly presenting soluble antigens to helper T cells.

Many inflammatory cells have a short half-life attributable to programmed cell death (apoptosis). Recent investigations demonstrate that the presence of inflammatory cells within tissues might not be entirely due to the continual attraction of these cells from the vasculature, but a prevention of apoptosis (119). In the context of the epithelium, conditioned medium taken from cultures of human bronchial epithelial cells markedly enhanced the survival of neutrophils, macrophages, and eosinophils (120-122). These survival-enhancing properties have been attributed to epithelial cell release of granulocyte-macrophage colony-stimulating factor (CSF), granulocyte-CSF, or macrophage-CSF, demonstrating that the epithelium has the potential not only for attracting cells, but also for prolonging their survival once they arrive (120-122).

The endothelial-derived relaxing factor has been found to be secondary to the enzymatic conversion of arginine to citrulline, releasing NO (123). However, it has also become apparent that NO may have a number of other effects in addition to smooth muscle relaxation, including neurotransmission, cytotoxicity of microorganisms and tumor cells, modulation of inflammatory cell chemotaxis, or alteration of enzyme activities (123). In the context of the lower respiratory tract and inflammation, neutrophils, macrophages, and bronchial epithelial cells have all been demonstrated to release NO under stimulated conditions, which suggests that a complex interplay may occur between epithelial cells and inflammatory cells via NO production and interaction with cell-associated proteins (123). Nitric oxide is also a potent bronchial vasodilator in animal airways (124). Nitric oxide-mediated dilatation of bronchial blood vessels could account, at least in part, for the bronchial edema that occurs during inflammation. In support of this concept, NO inhibitors reduce neurogenic plasma exudation in guinea pig airways (125).

Epithelial cells may also down-regulate inflammatory processes. Transforming growth factor- β (TGF β) is present in the epithelial lining fluid of the lung and is present in the epithelium of injured lung (126,127). In addition to its important effects on matrix production, TGF β has anti-inflammatory properties, such as the ability to inhibit IL-2-dependent proliferation of T cells (128), and also inhibits cytokine production by macrophages (129). Several cell types in the lung have been described as producing TGF β , including epithelial cells (130-132) and macrophages (133).

Other mediators with anti-inflammatory properties include PGE₂ and IL-6. Prostaglandin E₂ has a number of anti-inflammatory effects, including reduction in the production of neutrophil chemoattractants by macrophages (134). Interleukin-6 is capable of reducing inflammation in several models, including an *in vivo* model of pulmonary inflammation (135). In the context that IL-6 also has well-documented pro-inflammatory effects, such cytokines may be bifunctional, with differing activities depending on the progression of the inflammatory process.

Inflammation in the airway is a complex process that is regulated by many mediators. The capacity of airway epithelial cells to produce multiple mediators of inflammation suggests that these cells must be considered potentially important in regulating airway inflammation. However, the capacity of the epithelial cells to produce inflammatory mediators *in vitro* does not necessarily imply that these cells release the same mediators in any given pathological condition. Undoubtedly, the role of these cells in specific disease states awaits further study.

Repair

Direct injury of epithelium is a major feature of several airway disorders, including asthma and chronic bronchi-